PROFESSOR: We're going to get started. And today we're going to move forward with the protein degradation module, and looking at the degradation chamber of E. coli, ClpXP.

So this is a degradation machine. And effectively, what we see in this case, and as mentioned in lecture last time, is that there are gigantic chambers that isolate protease active sites. And so we're going to examine this particular machinery as a paradigm here.

So the Clp system was first identified in E. coli, and it's highly conserved. And what we'll see is that there's encapsulation of an active site in a large degradation chamber.

So there's two components, ClpX and ClpP. And so we're going to look at both of these components individually, and then see how this machine works. And so ClpP is the proteasome. And it's a serine protease. So we talked about serine proteases last time. So there's the catalytic triad of serine, histidine, and aspartate. So there's formation of a covalent acyl-enzyme intermediate. We learned that the serine residue is the active site nucleophile.

And what we're going to see is that this degradation chamber has 14 active sites.

And what this does is degrade proteins into short polypeptides. So those are peptides of about seven to eight amino acids.

And so if we consider a cartoon of the structure, what we find is that we have two back-to-back rings here. And each of these rings is a 7-mer. So we have two heptamers.

In terms of size, they're approximately 90 angstroms here and approximately 90 angstroms across. And then this region between the two rings is sometimes referred to as the equator.

So here we have two back-to-back rings. And these rings generate a chamber. And proteins upwards of about 70 kilodaltons can fit.

And so if we take this, looking at from the side, and just rotate 90 degrees to ask, what does it look like from the top? So this is a side view.

OK. What we see is that there is a very small pore here. So we have the seven subunits. And then in the center there is an axial pore. And this pore is small, about 10 angstroms in diameter.

So when thinking about that size, we need to think about the size of some large protein, right? If we have something on the order of 70 kilodaltons with a fold, that protein's not going to fit through this hole in that state here. OK? So it's too small for a big, folded protein.

But then basically, if we take this and, rather than just looking at the top, we cut through and ask what's going on in the interior, what do we see?

OK, so cut through.

What we see now is that there's a chamber of about 51 angstroms. OK, so this is the interior degradation chamber.

OK, so one question we're going to address as we move forward is that, how is it that a polypeptide gets through this hole into the degradation chamber that can accommodate a protein up to 17 kilodaltons? So small axial pore versus large degradation chamber here.

So we'll look at some structures of ClpP and then go on to ClpX. So what we're looking at here are effectively what I've drawn out in cartoon form on the board. So here we have the side view of ClpP. We have the top ring, the bottom ring, and here's the region between the two, the equator. If we look at the top, here they're describing the axial pore as a portal.

And here's the cutaway view. So this hole is very small. And if we look at the cutaway view, what we see is the degradation chamber here. And basically, the seven different serine protease active sites are shown here. And this is a side view cutting through the middle here. So we see that these serine protease active sites are down in this region here.

If we take another view and look at-- again, this is cutaway, so cut through the side view-- what we can look at here is the machinery in the active site. So we learned last time about the catalytic triad, with the aspartate, histidine, and serine. And in this particular structure there's a serine protease inhibitor bound to the serine side chain here. So that can serve machinery.

If we look at the structure of an individual ClpP subunit, those are shown here from several different organisms. What we see-- if we can think about this as the top part, and this is the bottom part of one ring-- we see there's a region with axial loops. There's a head domain and what's called a handle region. And the catalytic triad is located at the juncture of the head and handle region. And you can, again, look back to the cutaway views to orient that within the whole chamber.

These axial loops, we'll see, are important for interaction with ClpX. And we'll talk about that component of the machine in a moment.

Here, again, just structures of ClpP from various organisms, E. coli, Streptococcus, human. We see that they're all very similar here.

So what is ClpX? Moving forward.

OK. So ClpX is effectively an accessory protein. And in some respects we can think about it as a lid to the proteasome.

OK. It's a hexamer. So there's a mismatch here, in terms of the number of subunits in ClpP and ClpX. This is different from what we saw with GroEL, GroES, where they are both heptamers. ClpX is a hexamer. And it's an AAA-- so triple-A-plus unfoldase. It's an ATPase here.

And effectively, what we'll see is that ClpX has an important role as an accessory protein that unfolds the polypeptide that's destined for degradation by ClpP.

So it unfolds the polypeptide, and we're going to have to ask how as we go through.

OK. And in addition to unfolding, it also threads that polypeptide that's being unfolded in through the axial pores such that it can reach the degradation chamber, and threads it into the degradation chamber.

And so if we look at ClpX from a top view, again, we have a hole. And we have a 6-mer here.

So if we take a look, in this particular depiction what we're seeing are the top views and the side views. So here we have ClpP. Here we have ClpX. And what we want to ask is, how is it that ClpX binds to ClpP? And how is this mismatch in terms of the number of subunits

accommodated, right? So it's not that one subunit is precisely going to act with one, because we have six in-- throughout. Because we have six in ClpX and seven in ClpP.

And so if we think about how ClpX binds to ClpP, what we see is that we have the 6-mer. So we're looking from the side view. And there's some loops that are called IGF loops. So these are tripeptide motifs. And they're flexible. And these loops interact with hydrophobic regions of ClpP.

And this flexibility helps accommodate the six versus seven subunits.

So if we look here, here we see these tripeptide loops. And see, we're only seeing three. But there's one per subunit, so 1, 2, 3, 4, 5, 6 here. And then what's shown here in red are the hydrophobic regions of ClpP where these can bind.

And where do we see these regions on ClpP here? They're a bit removed from the axial pore here.

So how many of these loops are needed? Just to note, there's been studies done where these residues are deleted. And the question is, how many of these motifs are important for this protein-protein interaction? And what's been found in test-tube studies is that a minimum of two are required to get interaction between ClpX and ClpP here.

Yeah.

AUDIENCE: Is it known how many actually interact in vivo? Like, do all six interact at any given time?

PROFESSOR: I would presume so, but I don't know. Right? So we very much think of this as coming together as shown there. But don't know. Joanne, do you know? No. No.

I would say it needs to be pretty stable. Like, there's always dynamics. But as we see how this machine works, this hole is going to have to allow the polypeptide to thread through and get through that axial pore for the polypeptide to get in the degradation chamber. So you'd imagine you want that to be lined up quite well in order for it to be efficient there.

OK. So what are these triple-A-plus ATPases? This is a very important group of ATPases. So what the name means, ATPases associated with various cellular activities. And they're super-duper, given this. Hopefully everyone gets a triple-A-plus on the exam tonight.

The superfamily is involved in many cellular functions, and-- take a look. So, many diverse functions. Cell membrane fusion, trafficking of vesicles, cytoskeleton regulation, transport, organelle biogenesis, DNA replication, transcription regulation. And what we're really interested in here is protein degradation. So they come up in a variety of processes.

And although these processes are very different, all of these triple-A-plus ATPases share a common protein architecture. And I'll just point out that there's an ATP binding module. And some details are given here in terms of the motifs. And then really what we'll focus on, in terms of aspects of this course, is that they form oligomers that are ring- or cylinder-shaped. And they're all hexamers here.

And so, of importance to ClpXP, these ATPases have the ability to remodel conformation of macromolecules. And so here we're focused on unfolding.

Yeah.

AUDIENCE: [INAUDIBLE] question, but what are ATPases that aren't associated with cellular activities?

PROFESSOR: Well-- [LAUGHS] [INAUDIBLE]

AUDIENCE: Is this definition based on the architecture?

PROFESSOR: Strictly, yeah. I mean, there's--

GUEST SPEAKER: If you look at tRNA synthetases, they have ATPase activity. Hundreds of proteins have ATPase activities.

PROFESSOR: Right. So--

GUEST SPEAKER: They hydrolyze ATP to ADP and Pi.

AUDIENCE: Right, right, right. But that's a cellular activity, right?

AUDIENCE: So, like, what aren't AAA-plus ATPases?

PROFESSOR: Well, aminoacyl-tRNA synthetases are not triple-A-plus ATPase. What we'll see in terms of the non-ribosomal peptide synthetases, they're not these triple-A-plus ATPases. Right? So these-- I mean, yes, OK. All ATPases, the enzymes in a cell, it's going to have some role in a cellular activity, right? So maybe this name isn't very helpful. But what's common about all of these is that they share this common structural motif. They form these hexamers.

But within that, there's quite a bit of diversity, because they have all these different functions. So we can just see that here, to some degree. So-- oops. And there's a typo, which I'll fix before posting.

But if we take a look just at two examples here of different hexameric rings-- so these are two different triple-A-plus ATPases-- what do we see? So in common, they're both hexamers. In common, they both have an axial pore here. But we see different elements of secondary structure.

And granted, these are both depicted in a bit of a different way. But if we look here-- I mean, look. We have these alpha helical regions around the exterior that we don't see here. And in this view-- I show this particular one because it's depicted where the ATP is binding. So you can see the ATP binding to each subunit here. So, as shown, six ATPs bound.

So the structural diversity is quite tremendous. And here's just another example. So these are three different triple-A-plus ATPases of the Clp system. So we're going to focus on ClpX, but it's not the only one. And so what we're looking at here is ClpX. We have another family member, ClpA, and here, ClpB.

And so what we see is, subunit to subunit, whether it's X, A, or B, there's quite a bit of difference, right? So ClpX is the most simple, in terms of the architecture here, for that.

And so one thing people think about is, in terms of the different activities that have been associated with these different family members, how is it that these different structural features play a role? OK. Here.

So, coming back to ClpX and the depiction we saw before, ClpX is an unfoldase. And what's really a key point here is that ATP hydrolysis by ClpX is going to power conformational changes that allow for mechanical unfolding of this protein that's condemned for degradation by ClpP. And that's what's going to also allow for translocation of the resulting unfolded protein into the degradation chamber. So the action of ClpX is allowing that protein to fit through this axial pore and be threaded into the chamber.

So with that, what are the questions we need to address in thinking about how this macromolecular machine works? One, how are substrates recognized? So there's some certain group of proteins that are going to be degraded by this machinery. What is the

mechanism? How is it that ATP-dependent conformational changes of ClpXP drive unfolding and translocation? And what is the substrate selectivity? So that's where we're going to move forward with.

And so the first question we need to ask is, how are the substrates recognized by ClpX? OK? Here.

And so, what are possibilities?

OK. First, what we'll consider is a degradation tag.

So when I draw these cartoons, I'm only going to show one of the two rings for ClpP. It's not that it's only one. This is just for simplicity. But imagine that here we have X, here we have P. And we have some condemned protein, which I'll just draw as a circle. So the cell no longer wants this protein. It needs to go away.

And we can imagine, as one possibility, is that a degradation tag can be attached to this polypeptide. And what we find is that there's a particular tag called ssrA tag that is used to tag proteins for degradation by ClpXP. So we can think of this tag as a zip code. If a polypeptide gets modified such that this tag is appended, it's going to end up going to this degradation machine such that it gets degraded.

The tag is 11 amino acids. It's attached to the C-terminus.

OK. And the sequence is A, A, N, D, E, N, Y, A, L, A, A. And so what happens in this case, as shown-- we can imagine that this tag binds to the pore of ClpX directly. And the tag, when binding, enables translocation. So here--

OK. And this pore has what are termed pore loops that are involved in tag binding.

And in particular, there's a region, GYVG-- so, a four-amino-acid sequence-- that is thought to grip and drag the substrate. OK? Here. And of course it's not gripping like we would, but there's some interaction there happening that allows that to occur. So you'll see there's a lot of mechanical-type cartoons and language used in describing these machines.

OK. So what is another possibility?

So another possibility for how an ATPase could interact with a degradation chamber is that the

protein substrate binds to an extra domain attached to the ATPase.

OK. And I point out, this possibility is not for ClpX, but it's one to be aware of, because it can occur.

We saw some of those other ATPase are quite complicated. So in this case, imagine we have our ATPase, we have the degradation chamber. And this ATPase has some extra domain that effectively can bind the condemned protein and help deliver it to the pore here.

And just as a third possibility, and something that we'll see moving forward, is that there is involvement of an adaptor protein. So in addition to the ATPase and the degradation chamber, there's an adaptor protein that comes into play. So in this case, the protein--

OK, adaptor--

OK. And this protein helps direct it to the pore-- so, the condemned protein to the ATPase.

OK. So for instance, here we have the ATPase. Here we have the degradation chamber. And maybe there's some additional protein that facilitates getting the condemned protein to the ATPase.

And so something to keep in mind, and what we'll see with ClpXP, is that one and three are not mutually exclusive.

And there's an adaptor protein named SspB that can help deliver ssrA-tagged polypeptides or proteins to the degradation chamber here.

So we're going to think about these ssrA tags quite a bit. And something else to be aware of is just that these ssrA tags are not the only ways of tagging proteins for degradation. We're not going to talk about it in detail in this course, but you should be aware of something called the N-end rule. And this is really cool. So this rule basically states that a half-life of a protein is determined by its N-terminal residue here. And this can be called an N-degron. And these N-degrons are recognized by proteins such as ClpS and E. coli. And as a result, these proteins can get delivered to degradation machines.

So for instance, in addition to ClpXP, there's an ATPase, ClpA, that can associate with ClpP and be involved in degradation of polypeptides via this N-end rule. And in terms of the rule, depending on the identity of this N-terminal amino acid, it may be stabilizing or destabilizing, in

terms of protein lifetime. If you're curious to know more about that, we can refer you to some literature.

So here we have a cartoon looking at a native protein substrate that needs to be degraded. It's been modified with a tag. We have ClpX here. We have ClpP. Here's the tag. And in addition, we can have this adaptor protein SspB and the adaptor ClpS.

So let's think about this tag for a minute. And we need to think about this tag from the standpoint, one, of in vitro experiments, because we're going to begin to look at some experiments that were done to understand how this machine works. And we also need to think about this tag from the standpoint of the cell.

So if we think about an in vitro experiment where we want to study how ClpXP degrades some protein substrate, we can use this ssrA tag. And it's quite easy to attach 11 amino acids to some protein or polypeptide at the C-terminus. We can do that by protein expression, we can do that by chemical synthesis here. And so we're going to look at a number of experiments where this ssrA tag has been appended to certain model substrates, moving forward.

So what about in the cell? So when is this ssrA tag attached to a protein?

So are all proteins that need to be degraded destined to ClpXP? Just intuitively, what do you think? I see shaking heads, no. Right? There's many, many proteases around. So what proteins are destined for degradation by ClpXP? That's what we're going to look at, and how this tag is appended.

And so effectively, this ssrA tag, say, in E. coli, is used-- one, because protein degradation needs to be tightly regulated. But two, it's used for dealing with proteins that exhibited stalled translation.

So this discussion is going to bring us back to the ribosome here. So we want to ask what proteins in the cell are tagged with ssrA. How is the tag attached to the [INAUDIBLE] protein as well here?

This is just a cartoon showing an adaptor protein helping direct this tag to the substrate here. So we're going to just move forward to this slide. This tag is specifically added to proteins that are experiencing stalled translation. So it's estimated that on the order of 0.5% of E. coli translations result in ssrA tagging. And so this is thought to be one largely of quality control.

So you can imagine, if the ribosome stalled, there could be buildup of peptide products that aren't wanted. And the translation machinery could get blocked, and we don't really want that to happen here.

So here's our friend, the ribosome. And here's looking at the 50S ribosomal subunit. And we have a polypeptide emerging from the exit tunnel. So these should all be familiar at this stage.

And so what happens when this ribosome is trying to synthesize a polypeptide and it just gets stuck? So this ssrA tag is attached to the C-terminus of proteins. And as we're going to see, this occurs cotranslationally. And it's very, very interesting machinery.

So what we see here is that there's a new player we haven't yet seen. And this is called ssrA, or tmRNA, for transfer messenger RNA. And it's involved in attachment of this ssrA tag to polypeptides that are having stalled biosynthesis on the ribosome. And so this player acts as both a tRNA and an mRNA.

And we can take a look at the structure shown here. So here we have tRNA in the cloverleaf depiction, just an Ala-tRNA Ala. And if we take a look here, what do we see?

At this end we have a region of the tmRNA that looks like a tRNA. Right? Quite similar here to the [INAUDIBLE] prime end. And then we have this additional region. And then if we look down in here, what do we see? We see a region that, with a little imagination, we can think looks like mRNA. And if we take a look at the various codons, what we see is that the ssrA tag is encoded there, along with a stop codon. So effectively we have a tRNA look-alike. We have an mRNA look-alike that is encoding this ssrA tag.

So what happens? There's a partner protein called smpB just to be aware of. And in complex with smpB, it's actually EF-Tu that delivers this tmRNA to the ribosome here. So this is pretty interesting, just from the standpoint of what we know about EF-Tu. We don't have a typical anticodon here.

So how does that happen? We're not going to go into details, but it's something-- you know, curiosity should beg those questions. So what happens? We can look at this cartoon overview here.

And so the color coding within this is helpful, in terms of keeping track of pieces. But here we start with our stalled ribosome. So the mRNA is bound. We see there's a peptidyl tRNA in the

P-site. You know the polypeptide has a number of amino acids, and the A-site is empty. And for some reason, no new aminoacyl tRNA is coming in.

So the ribosome stalls. And as a result, this ssrA, or tmRNA, is recruited to this stalled ribosome. And so here we see the tRNA end in yellow, with the alanine attached. And here we have that region that's mRNA-like encoding the tag.

So this biomolecule gets recruited. And what do we see? It enters the A-site. So here we see the tRNA end in A-site, and we have the rest of the molecule here.

Then what? There's formation of a new peptide bond, so we have peptidyl transfer. We see that alanine is here. Look. That looks quite a bit like the hybrid states we talked about, where we're seeing these ends shift into the E-site, not shown, and the P-site here.

And then what happens? There's translocation and there's message switching. So the original mRNA gets kicked out. And what do we see? Now that mRNA-like region of the tRNA is in A-site here.

Then what happens after replacement of the mRNA? Translation can occur, which results in synthesis of the ssrA tag. So that's how this tag is attached to the C-terminus of the polypeptide. And elongation occurs until that stop codon in the tmRNA enters the A-site. And then peptide release occurs here.

So the result is a protein that has the ssrA tag attached to its C-terminus. And that protein will be directed to ClpXP. So, pretty cool. Yeah. I think so.

There. We don't ever leave the ribosome too much within these units. Just to point out, this tag is universal in bacteria. So here's just a table of phylogenetic distribution. You're not responsible for these details.

Yeah.

AUDIENCE: About the last slide-- so is the tag attached after it's stalled? Like, is the original protein completed? Or just the original mRNA removed and detached the tag? Or it's both?

PROFESSOR: Yeah. So what does the cartoon suggest?

AUDIENCE: It feels like it's already on the C-end here.

PROFESSOR: Well, the ribosome can stall at various points. So imagine you have a 100-amino-acid polypeptide that needs to be synthesized. The ribosome could stall after amino acid 20 or 40 or 60. It's not that the whole polypeptide has been synthesized and then this gets put on. It may be some fragment there for that. So, yes.

So in terms of this adaptor protein, I just want to make a note in terms of the adaptor. So these adaptor proteins can help with regulating the substrate specificity of triple-A-plus ATPases. And effectively, depending on the system and depending on the adaptor, it may enhance or it may inhibit degradation here. So it's a case-by-case basis.

This SspB shown in the cartoon here is a dimeric adaptor for XP, and it promotes degradation of certain substrates. And effectively, it enhances recognition of this tag by the machinery such that the degradation rates are enhanced. So it's not that it's required. It's just helpful, and accelerates the process.

So just an interesting observation regarding SspB-- it can be co-purified with ribosomes here. And in terms of its structure, it has some resemblance to known RNA-binding proteins. And this resemblance has begged a question, does SspB itself help with linking protein synthesis and protein degradation? So is it possible that SspB could help promote binding of ClpX to polypeptides before full release to the ribosome? That's something people have wondered about.

And initially this protein was classified as a stringent starvation protein. That's where the name comes from. So if we just take a quick look at its structure here, what do we see? So here is SspB. And then here we have structures from ribosomal proteins.

And so in SspB, ClpX binds on this side. And effectively, here we look at the ribosomal proteins that bind RNA. And they have these RNA-binding sites there. So there's some similarities in terms of the alpha helix, in terms of the beta sheets here.

And also, I'll just note, in terms of SspB and the ssrA tag-- so if we take this tag--

So this is our ssrA tag here. What's found is that ClpX recognition is on this end and SspB binding is on this end here.

So in different points. So what this indicates here is that SspB and ClpX can bind

simultaneously.

OK. But this is small, so we can expect that there's some clash here for that.

So where we're going to close is just looking at an overview as to how this machine works, and the model that then, starting on Friday, we'll look at experiments that were designed and performed to inform this model.

So if we look at this in one type of cartoon, what are the stages? So we can think of three as depicted here, where there's some sort of initial recognition. So the ssrA tag of this condemned protein binds to the axial pore of ClpX. And this process does not require ATP hydrolysis.

So here we see a folded substrate. This degron is another word for one of these tags, ssrA tag. So we see there's recognition here.

Then what happens, ClpX unfolds this substrate. So somehow it has to grip and pull and apply a force that unfolds the polypeptide, and threads that unfolded polypeptide into the degradation chamber. So, you know, kind of this pulley system is shown here. This chopper-type thing is shown here. You can use your imagination in this unit for how to depict this machine. So we see that the polypeptide is being unfolded and threaded through ClpX into this chamber, where it gets chopped up by the serine protease active sites here.

So for unfolding and translocation, ATP is needed. ClpX is hydrolyzing ATP to allow this to occur. In the degradation chamber, this degradation part is independent of ATP. Right? The serine protease doesn't need that here.

So how can we kind of break this up further into a model that we can test? What I present here is the working model. And just note, the orientation is flipped here. So we have ClpX on the bottom and ClpP on top.

So what happens here? We can look at this in terms of five steps. And we can begin here, with binding. So this ssrA-tagged protein needs to bind to ClpX. And that binding is associated with a dissociation constant, or Kd here.

What do we see? After binding we have a second step, which is denaturation. So the polypeptide becomes unfolded. And that's defined by a rate constant for denaturation, as shown here.

If we look next, we have translocation. So this polypeptide is moving through ClpX into the degradation chamber. And this is also associated with the rate constant-- so, rate constant for translocation. And both of these steps require the use of ATP.

Once this polypeptide is in the chamber, we have step four, which is degradation. And again, we have k deg. This is fast. And then in this last step here, there's some release. So somehow these polypeptide fragments need to be released from the chamber.

AUDIENCE: Is ClpP still a dimer at this point?

PROFESSOR: Yes, yes. So often the cartoons are drawn just showing one of the heptamers. But think of it as a dimer, with these two back-to-back rings here for that.

Right. So we have five steps here. Each one of these steps has a rate constant. And one question we want to ask with this is, what is the rate-determining step? And the quick answer where we'll end today, and as indicated in this overview, is that degradation is fast relative to denaturation and translocation.

And there should be an intuitive aspect to that. We heard about last time how proteases give these tremendous rate accelerations. And if you have an unfolded peptide, those sites where cleavage will happen are going to be exposed there.

So what we're going to ask is, is it possible to make experiments, design experience, where we can separate the denaturation process from the translocation process and analyze those-- and in the process of doing so, ask, what is the ATP utilization for each step? And what is the role for ATP in this process?

And so on Friday we'll begin with discussing substrates, the design of substrates that have been used, to examine this model in more detail. Is there one question next?

AUDIENCE: I was just wondering if the degradation step also removes translational modifications, or [INAUDIBLE]

PROFESSOR: In the degradation step? That's going to depend. I mean, you can have different types of bonds with post-translational modifications, right? Right. So in the eukaryotic system, you have a post-translational modification to direct this condemned protein. And that machinery-- so they're ubiquitins, and you get this polyubiquitin tail. So you saw ubiquitin in recitation number

one. And the eukaryotic proteasome has the ability to chop those ubiquitins ends off for recycling there, in that. So that's one example.