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ELIZABETH NOLAN: We're going to continue where we left off last time. So briefly I'll make a few points about initiation of translation in prokaryotes. And then where we're going to spend the bulk of the time today is with a review of tRNAs and then discussing the aminoacyl-tRNA synthetases, which are the enzymes responsible for loading amino acids onto the three prime end of the tRNA.

And these points are important because these process has to happen in order for the amino acids to be delivered to the ribosome, which is where we'll go on Wednesday. So the first questions are, how does initiation happen? So how does this ribosome, 70S ribosome, get assembled with the mRNA and initiator tRNA bound? And then we're going to ask, how do we get an aminoacyl-tRNA, such that the amino acids can be delivered to the ribosome?

So first, for initiation in prokaryotes, there's a few steps to this process. We'll just look at these at a basically superficial level of detail. But recall that there are translation factors. And during initiation, there are three initiation factors-- so IF 1, 2, and 3-- that are required to help assemble the 70S ribosome here.

So first in terms of initiation, what happens is that the mRNA needs to bind to the 16S RNA of the 30S subunit. And so I point this out because at this stage in the process, the 70S ribosome isn't assembled yet. So we have the mRNA binding to the small subunit. And this process requires initiation factor 3.

And effectively what happens is that the mRNA has a region called the Shine-Dalgarno sequence in prokaryotes, which is the site of ribosome binding. And then upstream of that is a start codon that signals for the start of translation. So if we think about the mRNA of the five prime end, and somewhere there's a sequence that signals for ribosome binding.

OK, and then we have our start codon that signals the start of translation. OK. And so this gets translated here. OK, so this start codon pairs with initiator tRNA. And this initiator tRNA is special. One reason why it's special is because the amino acid attached is an N-

Formylmethionine OK.

So sometimes the initiator tRNA is called f-met tRNA f-met as an abbreviation there. So just as some overview here, what we're seeing in this alignment is a number of the ribosome binding sites, or Shine-Dalgarno sequences in prokaryotes. We have the start codon on that pairs with the initiator tRNA. And here's a schematic depiction of what I've indicated here on the board.

OK, so the mRNA binds to the 16S of the 30S subunit. So the 70S is not assembled at this stage. And IF3 is involved, as I said. The Shine-Dalgarno sequence determines the start site. And we determine the reading frame, as well. So here is just an indicating translation of a polypeptide.

What happens after that? So after that, it's necessary to assemble the 70S ribosome, have the initiator tRNA in the P site, and have the cell ready to go for translation. And here's just one cartoon overview that we'll use as a description of this process. OK. So what do we see? We've talked about this step so far. We see there's a role for initiation factor 1.

And in this cartoon, if we imagine the E site, the P site, and the A site, what we see is that IF1 is binding to the site of the ribosome. And one way we can think about this is that the initiator tRNA has to get to the P site. And so that region is blocked to facilitate the initiator tRNA getting to the P site.

OK, we see that initiator tRNA binding to the P site. And this happens via formation of a ternary complex with IF2 and GTP. So initiation factor 2 hydrolyzes GTP. There's an event that results in joining of the two subunits. And there has to be dissociation of these initiation factors for the ribosome to be ready to accept its first aminoacyl-tRNA in the A site.

OK, so the outcome of this process here is that we have an assembled 70S ribosome with the initiator tRNA in the P site. The A site is empty, so it can accommodate an incoming aminoacyl-tRNA. And the E site or exit site is also empty. So that's the main take home for initiation. And that's the extent to which we're going to discuss it within this class.

So in order to get to the elongation cycle, we need to get the aminoacyl-tRNA into the A site. And that's going to require the help of EF-Tu, so elongation factor Tu. Before we discuss how elongation factor Tu is going to help deliver that aminoacyl-tRNA, we need to talk about how we get the aminoacyl-tRNA in the first place.

So what is the tRNA structure, just as a review to get everyone up to speed. How are amino acid monomers attached to the tRNA? And how is the correct amino acid attached? So this is an aspect of fidelity, which came up as a concept last week in lecture. And so we'll look at the mechanism of aminoacyl-tRNA synthetases to see how is the correct amino acid attached, and then what happens if the wrong amino acid is selected. Are there mechanisms to correct that? And if it's not corrected, what are the consequences here?

So moving forward with that, we're going to focus on the tRNAs and addressing those questions. So just as a review, so we can think about tRNA secondary structure, which is often described as cloverleaf. So we have a five prime end. The tRNA has several arms. OK. So we have a D arm.

This arm here has the anticodon that pairs with the codon of the mRNA. We have a variable arm, this arm here. And we have this three prime end here, where the amino acids get attached. So this, in terms of base numbering, we have C74, C75, A76 here. OH. This is often called the CCA acceptor stem.

And the amino acids are attached here. I'm going to abbreviate amino acid as AA via an ester linkage. And these ester linkages are important for the chemistry that happens in the ribosome. OK. So we can imagine just if we have abbreviating the tRNA structure like this and if we think about the sugar of A76-- bless you.

OK. We have one prime, two prime, three prime here. This type of connectivity here. And this is abbreviated throughout as amino acid tRNA, aa in general terms, or the three-letter abbreviations, like what we saw for f-met tRNA f-met with the initiator tRNA here.

So here's a schematic of a tRNA secondary structure with a bit more detail than what I show you on the board. And something we need to keep in mind is even though we often draw the tRNA in this cloverleaf type depiction, it has tertiary structure. And so it's very important to think about this structure as we think about how the tRNAs enter the various sites of the ribosome. OK.

So this structure is L-shaped. And I like this depiction here because regions of the secondary structure are color coded with the corresponding regions of this tertiary structure here. OK, so we see the shell shape of an L, rather upside down here, where we have the CCA acceptor stem over here and the anticodon arm and anticodon region down here.

So what is a consequence of this structure? The tRNA is quite narrow. So we're thinking about 20 to 25 Angstroms in width. And if we think about this in the context of the ribosome and the peptidyl transferase center, 3 tRNAs need to fit into that catalytic center during the elongation cycle. So it makes sense that they're relatively narrow. This allows three to fit there.

So as we think about the translation process and also think about some of the translation factors, we want to keep this type of structure in mind here. Here's just another view of that, with some additional descriptions of the overall structure. And this includes the numbering of the tRNA bases within that structure here.

Just a point to make, this won't be a major focal point in the course, but do keep in mind that tRNA contains many post-transcriptionally modified bases, so you'll see an example of that in problem set one. Up to 25% of the bases can be modified. Typically, we see about 5% to 20% of them modified here. OK, you're not responsible for these structures, these modified structures, in the context of this class.

So the key question for today is how are amino acids attached to the tRNA, as shown here? And in order for that to happen, there's a family of enzymes called aminoacyl-tRNA synthetases, or abbreviated aaRS. OK, so this name tells you right away, synthetase, that these enzymes use ATP. And these enzymes catalyze the attachment of amino acids to the three prime OH, or sometimes two prime OH, of the tRNA here for that.

And so we're going to consider this overall reaction. And then we're going to think about the reaction mechanism and experiments that were done to give support to the mechanism that we see. So all aminoacyl-tRNA synthetases require ATP and hydrolyze ATP to AMP and PPI. And so they catalyze this overall reaction where we have an amino acid monomer. We have the tRNA that encodes this-- that is for this amino acid. ATP to give us the aminoacyl-tRNA AMP and PPI.

So if the ATP is being hydrolyzed to AMP and PPI, what phosphate is being attacked? So we saw on Friday there's the alpha, beta, and gamma phosphates of ATP. OK, pardon?

AUDIENCE: Beta.

ELIZABETH NOLAN: Beta. Any takers?

AUDIENCE: Alpha?

ELIZABETH NOLAN: Any takers? Gamma? Yeah, so it's alpha. If you're getting AMP, it's attack at alpha. If you're getting ADP, it's attack at gamma here. OK, so P alpha is next door to the ribose of the nuc-- there. Yeah. OK.

So if we consider this overall reaction, how does it work? Just before that, one other observation I just want to point out, if we're thinking about these enzymes and asking what is it that they recognize of the tRNA, so we have the anticodon. And that goes in hand-in-hand with the identity of the amino acid. Just keep in mind that it's not just the anticodon.

So here we're seeing an example of an aminoacyl-tRNA synthetase with its tRNA bound. And we see that there's many contacts between the tRNA and this enzyme here. OK, so here we have the amino acid end, the anti-codon end, and all throughout here. So what is the mechanism to get us where we need to go? We have our overall reaction that I'll put up on the board, just to keep it straight as we move forward.

So amino acid plus ATP plus the tRNA for that amino acid. Aminoacyl-tRNA synthetase to give us the aminoacyl-tRNA plus AMP plus PPI. So let's consider a mechanism. This is going to be a two-step mechanism. And so in the first step of this mechanism, we have the amino acid plus ATP.

And we have formation of an OAMP intermediate. Plus PPI here. So this intermediate is called an amino adenylate. Adenlyate because adenosine here. And we need to think about why this intermediate might form. Why would we propose this in a mechanism?

And then in step two-- we'll come back to that in a minute-- we can take our amino adenlyate, have our tRNA, this is the three prime end here. We can have attack with release of AMP. OK, so here we have the ester linkage at the three prime end, like what we see on that board here, to give us our aminoacyl-tRNA.

OK, so we see in step one, there's formation of this amino adenylade intermediate. And in step two, there's transfer of the amino acid monomer to the three prime end of the tRNA here. So why might these enzymes go through that OAMP intermediate? What needs to happen for this chemistry to occur?

AUDIENCE: You need a more activated reading group to have that acyl substitution form an ester from a carboxylate.

ELIZABETH NOLAN: Right. We need to activate the CO2H group there. So this affords that. So what might be another possible mechanism, right? Imagine you're the experimentalist and you've combined your eighth amino acid ATP tRNA and this enzyme you've isolated in a test tube. And you see you've got this as a product. And this as a product.

And you're wondering how did we get from reactants to products? This is one possibility. Maybe there's also a possibility of a concerted mechanism where there's no intermediate like the one I'm showing you here. These are just things to keep in mind when thinking about reactions. This two-step mechanism is the accepted mechanism for the amino aceyl tRNA synthetases. And so what we're going to think about are what are the experiments that were done to support this mechanism here.

So what are the things we need to think about? And so we're going to think about this by examining one aminoacyl-tRNA synthetase as a paradigm. And this is the one for a isoleucine here. OK, so what are the experiments that need to be done to characterize this reaction and determine mechanism?

OK. So one thing we need to confirm is reaction stoichiometry. So there's a stoichiometry up in what I've written above. But experimentally, that needs to be determined. So one, reaction stoichiometry. And so how can we think about this? We can think about the equivalence of the amino acid. So in this case, isoleucine. How many equivalents of isoleucine?

And presumably, this isoleucine binds to the enzymes. We can think about it of equivalence of isoleucine bound. And we also see that ATP is consumed, right? That's hydrolyzed to AMP and PPI. So how many equivalents of ATP are consumed in this reaction? What else do we want to know? We need to know something about kinetics.

So what are rates of formation? What is the rate of formation of the product, the aminoacyl-tRNA, and since I've told you this intermediate forms, what is the rate of formation of the intermediate? And since this is an intermediate, it's something transient. So we need to think about how are we as experimentalists going to detect this intermediate over the course of this reaction. It forms and decays in order to get product here.

So rates of formation. And so we have formation of our product, which in this case-- and then formation of the intermediate, which I'll just abbreviate Ile-AMP. And what else would we like to know?

We can figure out how, in addition to rate of formation of the product and the intermediate, we can think about the rate of transfer of Ile from the intermediate to the tRNA. So what this tells us is that we need a way to look for or detect the intermediate. Here.

So imagine let's just have a hypothetical situation. If we find the intermediate, that tells us something about the reaction. If we don't find the intermediate, what can we conclude? Pardon?

AUDIENCE: That there was no intermediate?

ELIZABETH NOLAN: So that's one possibility. Are there other possibilities if our method doesn't let us detect the intermediate?

AUDIENCE: Second step is to test.

ELIZABETH NOLAN: Can it be hard to detect an intermediate? It can be very hard, right? So they don't always-- there aren't around all the time very much or in very abundant quantities. So if it's not detected, could it be there? Yeah, it might be there. And the method just didn't allow for it to be seen. So you always need to keep that possibility in mind. This will be a case where there is a robust method that allows us to detect this type of intermediate. But always keep that in mind.

OK, so first thinking about reaction stoichiometry. We're not going to go over the experiments that were done to define this. I'll just tell you some facts that result from some experimental studies. So this isoleucine aminoacyl-tRNA synthetase binds 1 equivalent of isoleucine as indicated in the overall reaction. And it consumes one equivalent of ATP, also as shown in this overall reaction, to make one equivalent of the aminoacyl-tRNA. OK and these stoichiometries were determined experimentally.

So now we need to think about points two and three to characterize the reaction kinetics. So what experiments were done? So there are several different sets of experiments, some of which we're familiar with from 7.05 or 5.07 and others that will be new and presented in more detail in recitation this week and next week. So we can imagine doing steady state kinetic experiments, as well as pre-steady state kinetic experiments. And the general aims here are, one, to determine the rate of aminoacyl-tRNA formation, to determine the rate of amino adenylate formation, so this intermediate-- and again, we need a method to detect the intermediate.

And at the end of the day, we'd like to know what is the rate determining step. So a method

that is commonly employed for these types of studies involves the use of radioactivity. And we'll just go over a few points about radioactivity now to help with understanding these experiments. And you'll hear more about this method in recitation this week.

So the experiments I'm going to tell you about are going to involve the use of radio isotopes like C14, P32. And the question is, why do we like to use radio isotopes in biochemical experiments? And they're really excellent probes. It's the bottom line. And one reason for that is that if you can use a radio isotope like C14 or P32, it's introducing minimal perturbation into your system.

So you're not needing to attach a fluorophore whether it be a small molecule or a protein. You're not modifying the structure of a component of your system. So the overall size and the chemical properties are maintained when you use different isotopes of the same element. And some of the ones we'll see today are, for instance, C14 labeled isoleucine, P32 labeled ATP.

They have the same chemical properties as the unlabeled forms, and same size. The other point to make is that we can detect very small amounts of radioactivity in a sample. And you'll see some of those calculations and how to do them in recitation this week. So we can detect small amounts, and that's good for looking for something like an intermediate. And there's readily available techniques for quantifying radioactivity in a sample.

So if you see nomenclature like this, the NX nomenclature indicates the radioisotope in this sample. And I'll just say in passing here, we all know the isotopes are atoms bearing the same number of protons but different numbers of neutrons. And radioactive isotopes have an unstable nucleus, which means there's a radioactive decay. And typically-- well, we often use beta emitters in biochemical studies. And that's what you'll see today.

So what are some of the experiments? We're first going to consider looking at the steady state kinetics to ask what do we learn in the steady state. So from our steady state experiments, we're able to get our Kcat and our Km and the catalytic efficiency, which is the Kcat over Km.

We're going to compare our Kcat values or turnover today. So experiment one is to monitor formation of product. So how is this done? This reaction is done by taking C14 labeled isoleucine and unlabeled tRNA and watching for transfer of that radio label to the tRNA.

And so what comes from these studies is a Kcat on the order of 1.4 per second. And now we have a way to detect this amino adenylate intermediate. And we'll talk about that assay in a

minute, after we get through this comparison. We do a steady state experiment to monitor formation of this amino adenylate intermediate.

And this assay also uses radioactivity. And it's called ATP PPI exchange assay. And we'll go over how this works in a minute. So the results of these experiments give a Kcat on the order of 80 per second. So what does this comparison tell you?

These values are quite different, correct? So we're seeing that this ATP PPI exchange assay is telling us that ATP PPI exchange, which is a measure of formation of this intermediate, is about 60-fold faster than formation of product here. That's an important observation to have.

So how are we going to figure this out? How are we going to see this intermediate? That's the question we need to ask next. And so we need to go over this ATP PPI exchange assay. And this is an assay that will come up again in module 4 when we talk about the biosynthesis of non-ribosomal peptides. So we'll return to this type of assay and data many times.

So the question is, if we have this reaction, OK, how do we detect this? OK, it's not so easy. And we need an assay. And this is some of the background towards the development of this assay. So we need to suppose that our amino acid and ATP react with the aminoacyl-tRNA synthetase in the absence of tRNA. And that's indicated by step one, more or less.

But that doesn't show it experimentally. So in the absence of tRNA, this amino acid and ATP react with the enzyme and they form the aminoacyl AMP intermediate and PPI. And they do this reversibly. OK, so the reversibility of this reaction is key for ATP PPI exchange to work.

So if this occurs and they do this reversibly, therefore we can deduce formation of the aminoacyl AMP. If we add radio labeled PPI, the amino acid, and ATP to the enzyme and we see that radio labeled phosphorus from the radio labeled PPI incorporate into ATP. That's only going to happen if this chemistry is reversible. And bear in mind, we can detect very small quantities with radioactivity. So it's not that it has to be reversible to some large degree. We're relying on the detection of this radio label.

So how does this work chemically? Let's take a look. OK, so imagine here we have our ATP. We have our amino acid. And we have our enzyme. And step one, we have binding. So there's some ATP binding site to the enzyme and some site for the amino acid to bind. And I'm leaving magnesium out of this depiction, but remember that magnesium and ATP come together. Now what?

Step two, OK, we're going to have a chemical step where we have formation of the amino adenylate and PPI. And they currently are bound to the enzyme. We have step three. So imagine in this step our PPI is released. And this is another key aspect of this assay.

So what does this mean? We now need to think about going backwards. If the PPI is released and we spike this reaction with radio labeled PPI and work our way backwards, will the radio label end up here in the ATP? OK. So this is going to be going backwards. We've left off with this enzyme with the amino adenylate bound. We have the PPI that was released.

And then we spike this reaction with our radio labeled or hot PPI. So then what happens? Step four, working backwards. Imagine that some of the radio labeled PPI binds. Then what? Working backwards another step. 32 P ATP and the amino acid.

And then we have release here. OK, so then the question is, can you detect this? And so if you can detect some incorporation of this radio label into the ATP, that indicates that this enzyme worked through that type of intermediate.

AUDIENCE: So are PPI not also sometimes [INAUDIBLE] and then if you had some competing hypothesis where it made ATP and ADP, then your PPI would maybe sometimes turn into just a single radio label phosphate that could then have the same reverse reactions as the [INAUDIBLE]?

ELIZABETH NOLAN: Yeah. So whether you initially end up with PPI or PI is going to depend on how the ATP is hydrolyzed. And so you could imagine maybe there could be some background ATP hydrolysis that gives ADP and PI in this type of assay. That's something you always need to look out for. For the purpose of this, let's assume that we're not having some background problem in terms of the ATP source, and also that the enzyme is specific in terms of what it's doing to the ATP. But yeah, certainly background ATP hydrolysis can be a problem.

So how will this be detected? And how will you know the radio label is associated with ATP and not something else in your mixture?

AUDIENCE: [INAUDIBLE]

ELIZABETH NOLAN: Pardon?

AUDIENCE: [INAUDIBLE]

ELIZABETH NOLAN: No. So we're going to look at the radioactivity. So this will come up more in recitation this week. But we need to be able to measure radioactivity by, say, scintillation counting here. But what's also needed is a separation because you need to know where that signal's coming from. You need to know it's coming from ATP and, say, not a background from however much of the PPI you introduced.

Or if you have no idea what's going on with your chemistry, maybe the data are going to tell you it's not this mechanism. So you need to have a separation. So how might you separate ATP from all of these other components?

AUDIENCE: Based on affinity column.

ELIZABETH NOLAN: Some affinity column. So I like the column. But we're not going to have some sort of tag on the ATP. That might be a problem for that enzyme. But your notion is correct in the sense that we'll use some sort of chromatography in order to separate. OK, so maybe HPLC, how many of you have used an HPLC or at least know what one is?

AUDIENCE: [INAUDIBLE].

ELIZABETH NOLAN: Right. So typically looking at UV vis. But you can imagine hooking up an HPLC to a detector that allows you to do scintillation counting and some sort of column that will allow you to look for ATP. Is all of the ATP going to be radioactive in this assay? No. So again, we can detect small quantities. And as long as there's a little bit of reversibility, we can see this here.

OK, so what's critical in this assay is the reversibility of steps 3 and 4. What would happen in this assay if the PPI is not released?

AUDIENCE: [INAUDIBLE].

ELIZABETH NOLAN: Right. Under the conditions, or if for some reason the PPI is not released, we're not going to see this exchange reaction. We're going to have a readout that doesn't give us this. Does that mean this didn't form? No. OK, so there's many caveats and details that you need to think through when thinking about a reaction and then the experiment is done to test this.

So in the case of these aminoacyl-tRNA synthetases, these ATP PPI exchange assays work well. And these assays can be used to get steady state kinetic parameters, to get Kcat, Km, Kcat over Km, which is where this type of value comes from, in this case here.

So back to these analyses up here, what they're telling us is that formation of this amino adenylate intermediate is about 60-fold faster than formation of the product. OK. And what we all want to recall when thinking about steady state experiments is that they're set up with a great excess of substrate and with the enzyme concentration. The reaction is zero order in respect to substrate. And you'll have some additional notes about that in your recitation materials this week for review.

So something else biochemists like to do when looking at reactions and understanding reaction mechanisms is to look in the pre-steady state. And this came up briefly in lecture 1 as a method. And again, you'll hear more about it in recitation over the next two weeks. In these experiments, the goal is to look at the very first, early moments of a reaction. And they're set up quite differently.

So limiting substrate is used. There's no turnover, so huge contrast to what we know about steady state experiments. And one of the goals is to look at the formation and consumption of intermediates here. So this type of chemistry often happens on a fast timescale. You can imagine millisecond timescale here, which means that we need a special apparatus that has fast mixing capabilities, because there's no way for one of us to do this on our own with our pipette.

And so the type of experiment or apparatus used is called a stop flow. And I just show one depiction of a stop flow apparatus here. You'll get some other variations on this theme in the recitation notes. OK, but effectively what happens is that you have two drive syringes, a and b, and each of these syringes will contain certain components of your reaction. And this stop flow has a drive motor and a stop syringe. And it effectively allows you to rapidly mix the components of these syringes in a mixer, shown here.

And then you either have some way to detect product-- so maybe if you can use optical absorption, you have a UV vis detector or a fluorescence detector. Or in other cases what you'll do is you'll punch the reaction at a certain time point. So you need a third syringe not shown here with a quencher. So you can imagine if you're working with an enzyme, maybe you quench by addition of acid or base, something that will denature and precipitate that enzyme.

And then you can take that sample and analyze it in some way that fits in terms of what you need to detect there. So this type of methodology was used in order to monitor transfer of

isoleucine to its tRNA. And so where we'll pick up in lecture on Wednesday is the design of that experiment in terms of what will we put in each syringe, and then what are the results of those experiments? And ultimately, what does that tell us about rates of transfer here? That's where we'll continue.