Lecture 25
4/14/04

References for unnatural amino acids:
Wang, Schultz, Rev. Chem. Comm. (2002) 1-11
Scultz Science (2003) 301, 964-7 (eukaryotes)

3 methods for the incorporation of unnatural amino acids
(1 *in vitro* and 2 *in vivo*)

1) Auxotrophs (*in vivo*) : unnatural amino acids must look like the natural amino acid to be charged onto the tRNA and to avoid editing (often done with fluorinated amino acids)

Example: fluorinated tyrosine- see lec 24 notes
Organism must also take up unnatural amino acid
Fool the tRNA synthetase, and load unnatural amino acid on $tRNA^{tyr}$
In all proteins, tyrosines are replaced by fluorinated tyrosines.

2) *in vitro*- unnatural amino acid at a single site, using the translation apparatus

Takes an army of people to make the charged tRNAs containing the unnatural amino acid, the extract that contains all of the factors, the ribosomes, the DNA with the appropriate non-sense codon etc, very time consuming and labor intensive- not widely used

Limited by the amount of protein produced (very low yields) and the amount of time to make all of the reagents.

See p13 of handout 3b
(most of this work done in the Shultz lab)
Replace codon for amino acid of interest with "nonsense" or stop codon.
Take tRNA w/ anticodon for "nonsense" codon, and synthesize this tRNA through *in vitro* transcription with T7 RNA polymerase. BUT, only synthesize tRNA-C at 3' end (leave off last two bases C-A with charged amino acid) – remember, bases are not modified in *in vitro* transcription and thus the efficiency of suppression by the charged tRNA may in part be compromised.
Make dCpA-unnatural amino acid and ligate it to the tRNA with T4 RNA ligase
Gives tRNA w/ nonsense anticodon, charged with unnatural amino acid
Then, *in vitro* translation
"gamish"- a mixture off all ingredients needed for translation- DNA with gene containing non-sense codon, ribosome, all elongation and release factors, etc… a big mess
make protein w/ unnatural amino acid inserted specifically at desired site!

Stop codon (UAA,UAG,UGA)
unnatural tRNA needs to out-compete release factor (RF-1 and RF-2 for binding to the stop codon)
Problems: only get a small amounts of protein
Has not been widely used because so technically difficult, takes a LOT of work

How large can the unnatural amino acids be? How large can the protein be? See handout of the number of unnatural amino acids successfully incorporated into protein using this technology. It is pretty impressive.

3) *in vivo* - unnatural amino acid incorporated at a single site

Develop a tRNA/RS pair (w/ stop anticodon) that only recognize each other and only react with unnatural amino acid of interest. Thus for each unnatural amino acid that you are interest in you need to evolve a separate RS/tRNA pair.

Then the RS can attach the unnatural amino acid to the tRNA

Brute force selection- evolve tRNA/RS for **EACH** unnatural amino acid you want to use

Put the genes for the tRNA and RS that have been evolved into the cell

The organism must also contain the gene of interest with stop codon inserted. Then feed unnatural amino acid to the organism. The unnatural amino acid must be taken up by cell and be non-toxic!

Get out protein w/ unnatural amino acid specifically located.

One example

unnatural amino acid= **benzophenone crosslinker**

Other ideas- fluorescent probe as unnatural amino acid, or a reactant group that could be attached to a fluorescent probe under mild conditions

This technique is still in the early stages

What is the scope of this method? Small and large proteins? Yields?

See p. 14 of handout 3b for a library of unnatural amino acids that have been incorporated using *in vitro* method (2)

This concludes Module 3!

Module 4 – Crypts and Chambers

Overview: What happens as the protein exits the ribosome?

a. spontaneous folding- but many proteins require chaperones (ATP)
b. membrane proteins and secreted proteins (or in eukaryotes proteins that need to be glycosalated) have a signal peptide at their N-terminus that is recognized by a SRP (signal recognition particle)-GTPase. This SRP with the N-terminus of the polypeptide interacts with the SRP-R (signal recognition particle receptor)-also contains a GTPase. The SRP-R is found in the ER membrane. The GTPases work in concert to transfer the growing polypeptide to a translocon in the ER membrane.
c. proteins made in the cytosol – may need to get into mitochondria (TOM (protein machine to transfer proteins across the outer mitochondrial membrane, TIM (tranport across the inner mitochondrial membrane)
d. what happens if proteins are misfolded?

A. Protein Folding

1. in vitro- "Anfinsen's hypothesis" – latest model for protein folding will be presented
- relationship of *in vitro* and *in vivo* folding
2. proteins (chaperone proteins) involved in *in vivo* folding

use of energy- ATP

B. Proteosome

Misfolding-> degradation (ATP)

Regulation-> degradation (ATP)

Ribosome is free in the cytosol, growing polypeptide comes out of the exit tunnel

Cartoon:

Spontaneous folding? (yes, can occur)

Proteins that facilitate folding (yes)

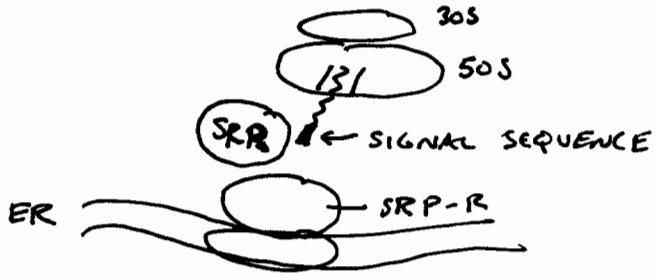
Proteins- membrane insertion into the ER; proteins that are secreted or need to be glycosylated all need to go into the lumen of the ER.

Hydrophobic sequence at N-terminus of a polypeptide (address code) interacts with SRP(marcromolecular machine)

SRP(signal recognition particle) has a GTPase and is composed of RNA (300 nts) and 7 proteins in mammalian systems. The equipment is conserved throughout evolution and is much simpler in bacterial systems.

SRP-R(signal recognition particle receptor)-also a macromolecular machine that has a GTPase and a translocon, allows protein to get into membrane or into the lumen of the ER

Cartoon:



See also p2 of handout 4a

Look at differenet SRP structures

E. coli is the simplest, mammalian system more complicated

P3 of handout 4a – Ribosome interacting w/ SRP- cryoEM

Cartoon- synthesis of information from many studies (xray, cryoEM, fluorescence measurements, GTPase assays

Nature (2004) 427, 808-14

1) SRP binds the signal sequence
2) Pauses- part of SRP folds back around the ribosome into the A site!
3) Promotes protein translocation through interaction w/ SRP-R and its translocon