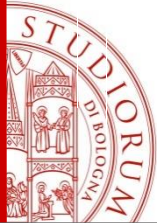


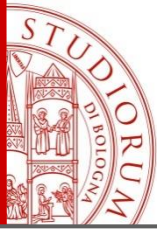
NUCLEIC ACIDS – BIOCHEMISTRY MODULE 2

- **Structure and Function of Nucleic Acids**
- **Genes and Chromosomes (brief summary)**
- **DNA Replication**
- **DNA Transcription**
- **Protein Synthesis**



EXAM PROCEDURE

- The final examination consists of a written exam, including Chemistry and Biochemistry parts.
- **Maximum duration of 50 min** (25 min for EACH part).
- The exam consists of true/false, single choice (3 choices), and open questions.
- The overall exam score is 33.00 points, equally divided into 16.50 points for the “Chemistry” part and 16.50 points for the Biochemistry module. **The exam is passed by achieving at least 9 points (out of 16.50) in each part.** The grade obtained (necessarily greater than or equal to 18) is calculated by summing the points obtained in each of the above-mentioned parts and rounding it to the nearest integer.
- The exam can be repeated at any exam session. No midterm exams will be scheduled.
- **The Chemistry module score, once accepted, must NOT be repeated.**



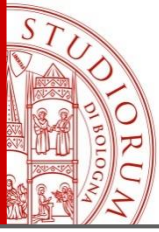
EXAM CONTENT

CHEMISTRY

- **8 true/false questions: 0.4 pts point/correct answer**
- **8 single choices; 3 options with 1 correct answer, 0.85 pts/correct answer**
- **2 exercises: 2.25 pts for each correct exercise**
- **1 open question: 2 pts**

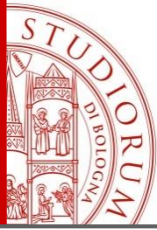
BIOCHEMISTRY

- **8 true/false questions: 0.4 point/correct answer**
- **10 single choices; 3 options with 1 correct answer, 0.85 pts/correct answer**
- **2 open questions: 2 pts and 2.8 pts, respectively.**



EXAM PROCEDURE

- **If a student obtains an overall score greater than 30.5 points (from 30.51), the examining commission will confer 30 cum laude (30L)**
- **If a student obtains 8.5-8.9 points (out of 16.5) in one of the modules, s/he will receive a final grade of 18.**



EXAM EVALUATION

Examples:

- Chemistry: 8 pts
- Biochemistry: 12.50 pts

---> FAILED

- Chemistry: 16.5 pts
- Biochemistry: 9.75 pts

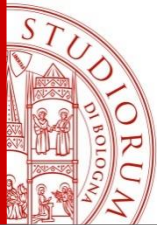
---> 26.25 pts ---> PASSED WITH 26/30.

- Chemistry: 16.5 pts
- Biochemistry: 8.5 pts

---> PASSED WITH 18/30.

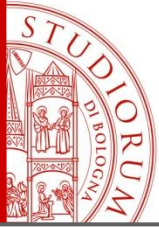
- Chemistry: 16 pts
- Biochemistry: 9.5 pts

---> 25.5 pts ---> PASSED WITH 26/30.



Exam dates

- **February 4th, 2025 - 09:30 at Aula magna DERMATOLOGIA** Just Chemistry exam (30 min)
- **February 18th 2025 - 09:30 at Aula magna MURRI**
- **March 6th 2025 - 09:30 at Aula magna MURRI**
- **June 10th 2025 - 09:30 at Aula magna DERMATOLOGIA**
- **June 24th 2025 - 9:30 at Aula magna DERMATOLOGIA**
- **July 15th 2025 - 9:30 at Aula magna DERMATOLOGIA**
- **September 16th 2025 - 09:30 at Aula magna DERMATOLOGIA**



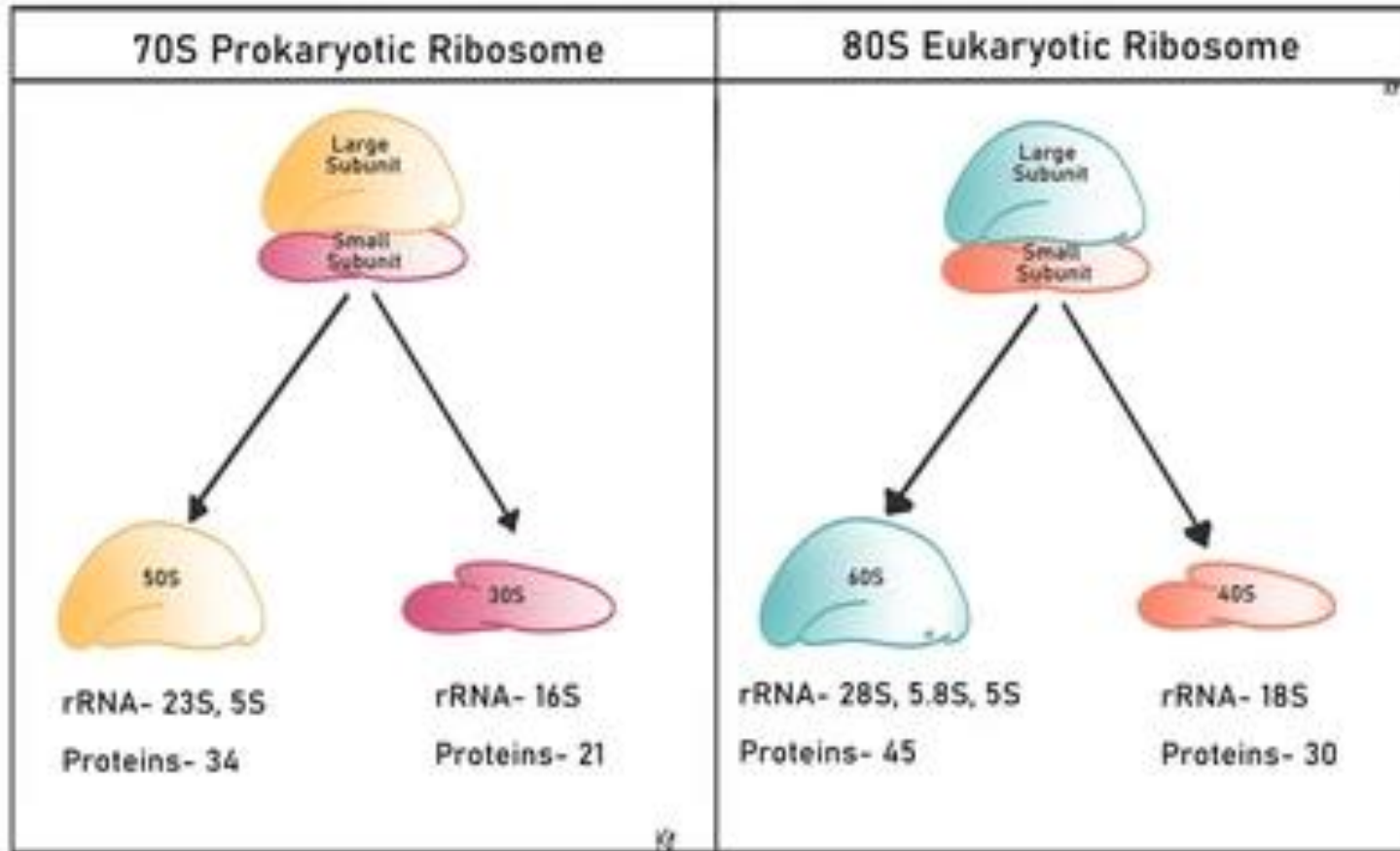
RIBOSOMES

Functions:

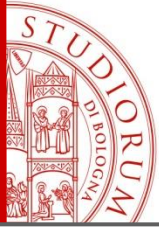
- Bind mRNA;
- Contain specific sites for aminoacyl-tRNA (there are 3: one for the incoming aminoacyl-tRNA A, one for the tRNA that carries the growing polypeptide chain P, and one for the tRNA that has just donated its amino acid group E);
- Facilitate interaction between non-ribosomal protein factors necessary for the initiation of protein synthesis;
- Catalyze the formation of the peptide bond;
- Are capable of translocating the peptidyl-tRNA with the nascent chain and mRNA to bind everything together.

RIBOSOMES

EUKARYOTIC AND PROKARYOTIC RIBOSOMES

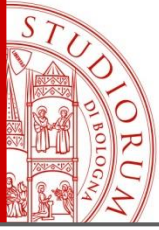


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RIBOSOMES

- In *E. coli* a ribosome has a diameter of approximately 25 nm and a mass of 2520 kD, composed of two unequal subunits that dissociate at magnesium concentrations below 1 mM.
- The 30S subunit consists of 21 proteins and a 16S rRNA, weighing about 930 kD.
- The 50S subunit contains 31 proteins and two rRNAs (23S rRNA and 5S rRNA), with a total weight of about 1590 kD.
- Ribosomes are primarily made up of RNA, accounting for about two-thirds of their structure, with around 20,000 ribosomes present in a single cell, making up about 20% of cellular mass.



RIBOSOMES

- Mitochondrial and chloroplast ribosomes are highly similar to prokaryotic ribosomes, reflecting their presumed prokaryotic origin. This similarity suggests that these organelles may have evolved from free-living bacteria that were engulfed by ancestral eukaryotic cells.
- In contrast, cytoplasmic ribosomes in eukaryotes are larger and more complex, classified as 80S ribosomes compared to the 70S ribosomes found in prokaryotes. Despite their differences in size and complexity, many structural and functional properties of eukaryotic cytoplasmic ribosomes remain similar to those of prokaryotic ribosomes.



PROTEIN SYNTHESIS

Genetic code

mRNA (4 Nucleotides) $\xrightarrow{?}$ aa sequence (20 Amino acids)

$4^2 = 16$ codon combinations
 $4^3 = 64$ codon combinations
= 61 coding for 20 amino acids
+3 nonsense (stop codons) (UGA, UAG, UAA)

Nobel Award for Medicine in 1968 for the interpretation of the genetic code and its function in protein synthesis



Robert W. Holley

Cornell University
Ithaca, NY, USA



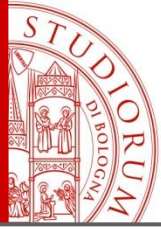
Har Gobind Khorana

University of Wisconsin
Madison, WI, USA



Marshall W. Nirenberg

National Institutes of Health
Bethesda, MD, USA



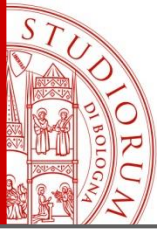
PROTEIN SYNTHESIS

Genetic code

To transition from the sequence of nitrogenous bases in DNA to the sequence of amino acids in proteins, two essential components are required:

A Genetic Code: This code must be capable of encoding all 20 amino acids using the four characteristic nitrogenous bases of nucleic acids (adenine, thymine, cytosine, and guanine). The genetic code is organized into triplets (codons), where each triplet corresponds to a specific amino acid.

A Translator: This is a molecule that links the genetic code to its corresponding amino acid. The translator in this context is transfer RNA (tRNA), which carries amino acids to the ribosome during protein synthesis.



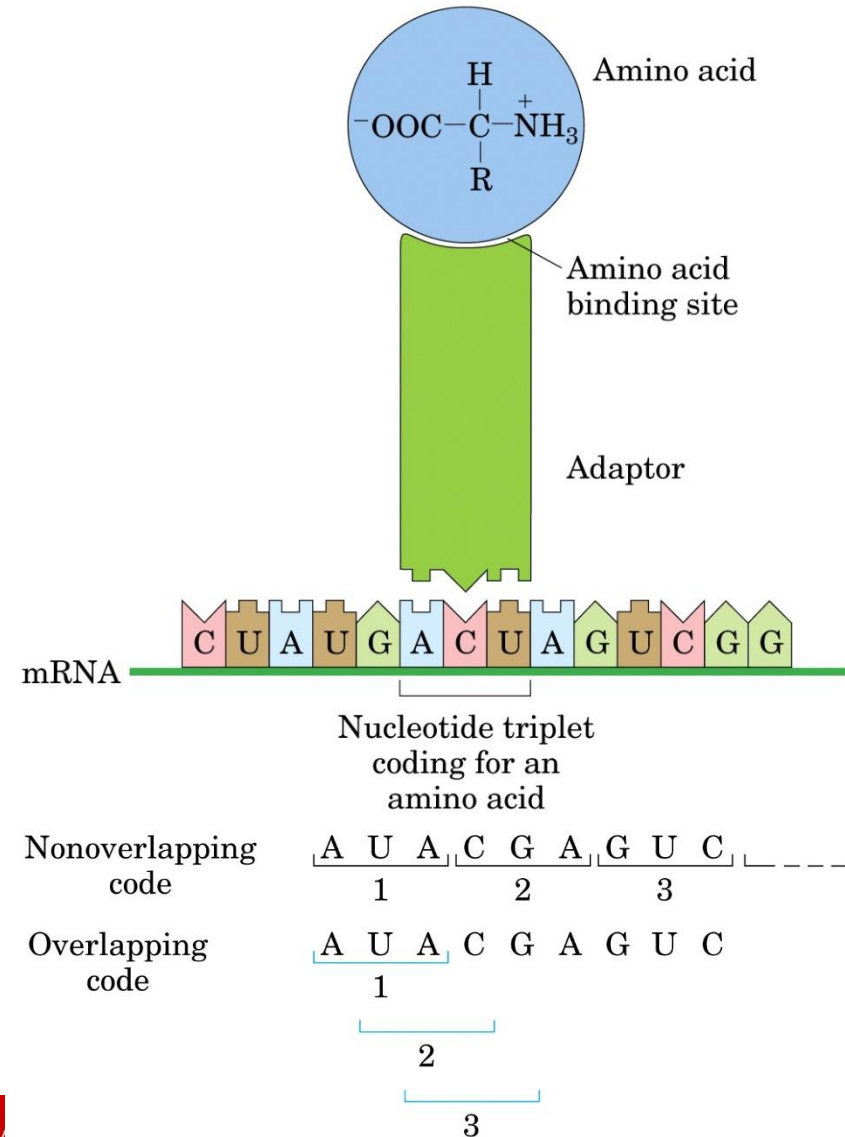
PROTEIN SYNTHESIS

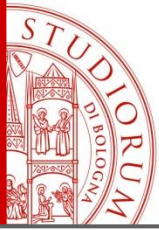
Genetic code

The transition from a four-letter code (nucleotide bases in DNA) to a twenty-letter code (amino acids in proteins) is facilitated by reading nucleic acids according to a triplet code.

mRNA and tRNA: Messenger RNA (mRNA) does not directly recognize amino acids; instead, it is transfer RNA (tRNA) that carries specific amino acids. Each tRNA molecule is linked to one amino acid and recognizes the corresponding codon on the mRNA.

Codon-Anticodon Interaction: tRNAs recognize the coding triplet on the mRNA via a complementary triplet known as the anticodon. This interaction ensures that the correct amino acid is added to the growing polypeptide chain during protein synthesis.





PROTEIN SYNTHESIS

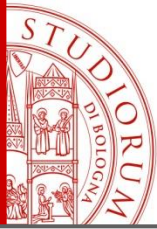
Characteristics of the Genetic Code

	1 ^a posizione			2 ^a posizione			3 ^a posizione		
5'	U	C	A	G	3'				
U	UUU Phe	UCU Ser	UAU Tyr	UGU Cys	U C A G				
	UUC Phe	UCC Ser	UAC Tyr	UGC Cys					
	UUA Leu	UCA Ser	UAA Stop	UGA Stop					
	UUG Leu	UCG Ser	UAG Stop	UGG Trp					
C	CUU Leu	CCU Pro	CAU His	CGU Arg	U C A G				
	CUC Leu	CCC Pro	CAC His	CGC Arg					
	CUA Leu	CCA Pro	CAA Gln	CGA Arg					
	CUG Leu	CCG Pro	CAG Gln	CGG Arg					
A	AUU Ile	ACU Thr	AAU Asn	AGU Ser	U C A G				
	AUC Ile	ACC Thr	AAC Asn	AGC Ser					
	AUA Ile	ACA Thr	AAA Lys	AGA Arg					
	AUG Met	ACG Thr	AAG Lys	AGG Arg					
G	GUU Val	GCU Ala	GAU Asp	GGU Gly	U C A G				
	GUC Val	GCC Ala	GAC Asp	GGC Gly					
	GUA Val	GCA Ala	GAA Glu	GGA Gly					
	GUG Val	GCG Ala	GAG Glu	GGG Gly					

Degeneracy: More than one codon can specify the same amino acid.

Non-ambiguous: Each triplet codon codes for only one specific amino acid.

Universality: The codons corresponding to the 20 amino acids are largely consistent across different species, with some exceptions in mitochondria.



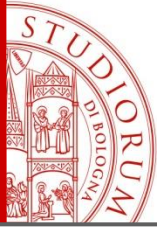
PROTEIN SYNTHESIS

Genetic code

Degeneracy of the Genetic Code

U, C, A, and G can make 64 3-base combinations (4^3), but there are only 20 amino acids plus stop to code for.

Number of codons for each amino acid	Number of amino acids (or STOP)	Examples
1	2	Methionine, Tryptophan
2	9	Phenylalanine, Tyrosine, Histidine, Glutamine, Asparagine, Lysine, Aspartate, Glutamate, Cysteine
3	2	Isoleucine, STOP
4	5	Valine, Proline, Threonine, Alanine, Glycine
6	3	Leucine, Arginine, Serine



PROTEIN SYNTHESIS

tRNAs

tRNAs have a characteristic cloverleaf structure with specific regions that facilitate their function, including an acceptor stem for binding the amino acid and an anticodon loop for codon recognition. Common characteristics include:

5' Phosphate Group: Each tRNA molecule has a phosphate group at the 5' end.

Accepting Stem: A 7-base pair (bp) stem known as the acceptor stem, which is involved in binding to the amino acid.

D Arm: A stem of 3-4 bp plus a loop of 5-7 bp referred to as the D arm.

Anticodon Arm: A stem of 5 bp that ends with a loop containing the anticodon, known as the anticodon arm.

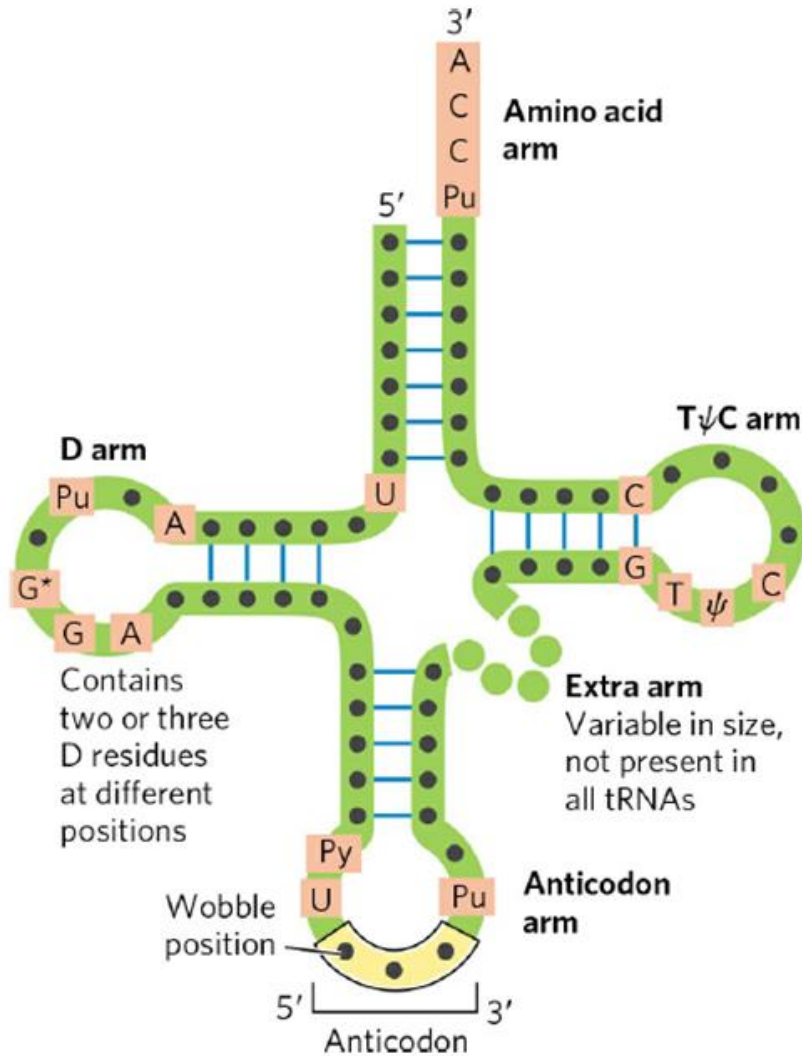
T Arm: Another 5 bp stem that terminates with a T loop, referred to as the T arm.

CCA Sequence: A -CCA single stranded sequence at the 3' end with a hydroxyl (OH) group where the amino acid attaches.

Variable Region: A variable region corresponding to the T arm.

PROTEIN SYNTHESIS

tRNAs

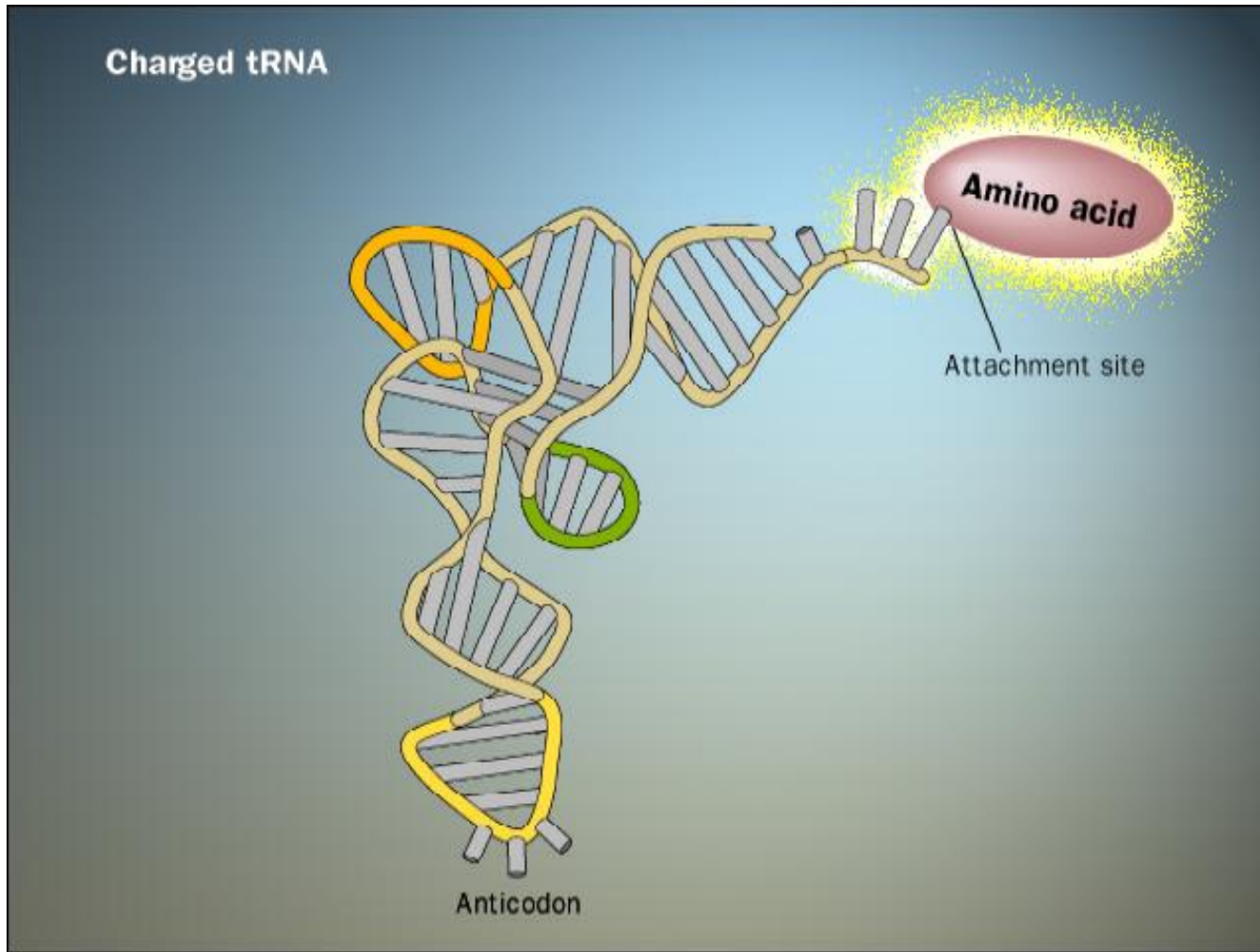


The D arm contains two or three D (5,6-dihydrouridine) residues, depending on the tRNA. In some tRNAs, the D arm has only three hydrogen-bonded base pairs. Pu represents purine nucleotide; Py, pyrimidine nucleotide; ψ , pseudouridylate; G*, guanylate or 2'-O-methylguanylate.

PROTEIN SYNTHESIS

tRNAs

The amino acid binds to the 3' end of the tRNA (-CCA sequence)





PROTEIN SYNTHESIS

tRNAs

Amino acids are linked to their corresponding tRNA by **aminoacyl-tRNA synthetases**.

Activation of Amino Acids

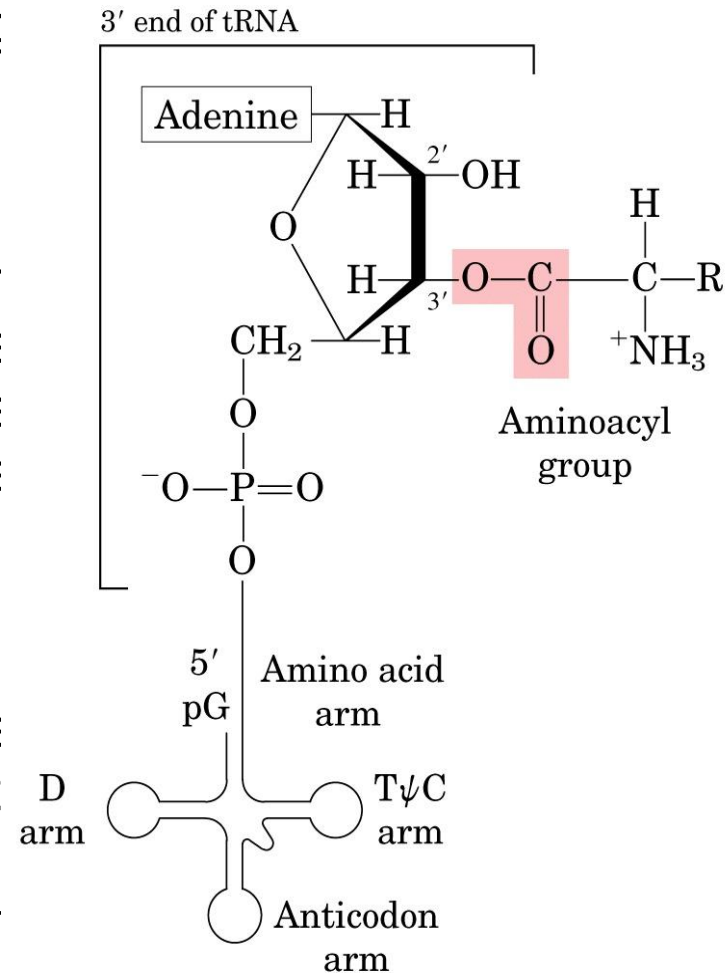
This activation involves the formation of an **aminoacyl-adenylate complex**, where the amino acid is linked to adenosine monophosphate (AMP) derived from ATP. The reaction can be summarized as follows:

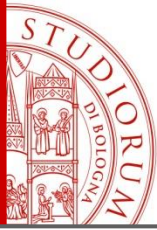


Classes of Aminoacyl-tRNA Synthetases

Class I Synthetases: Typically recognize the anticodon and bind the amino acid at the 2' position of the ribose in the A of tRNA.

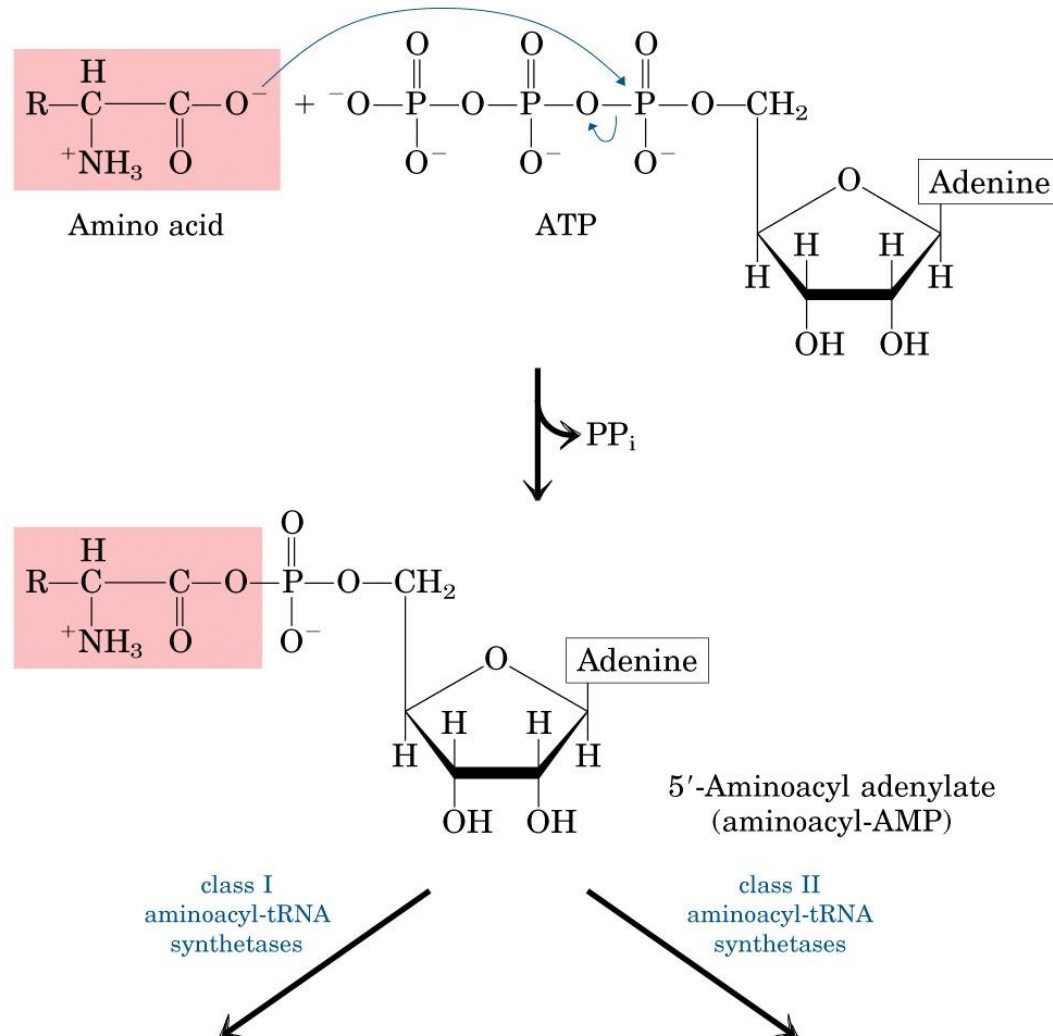
Class II Synthetases: Bind the amino acid at the 3' position of the tRNA and do not typically recognize the anticodon but focus on other structural features of the ribose in the A of tRNA.





PROTEIN SYNTHESIS

tRNAs





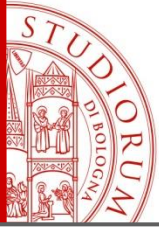
PROTEIN SYNTHESIS

tRNAs

table 27-8

Two Classes of Aminoacyl-tRNA Synthetases*

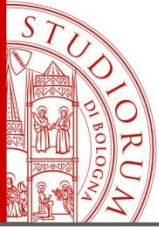
Class I	Class II
Arg	Ala
Cys	Asn
Gln	Asp
Glu	Gly
Ile	His
Leu	Lys
Met	Phe
Trp	Pro
Tyr	Ser
Val	Thr



PROTEIN SYNTHESIS

Aminoacyl-tRNA Synthetases:

- Aminoacyl-tRNA synthetases exhibit two levels of specificity:
First Level: Specificity in forming the correct aminoacyl-adenylate.
Second Level: Specificity in binding to the correct tRNA molecule.
- **Proofreading Mechanism:**
 1. the wrong aminoacyl adenylate enters the proofreading site and is hydrolysed.
 2. the amino acid is loaded onto the tRNA but the aminoacyl tRNA complex assumes the wrong structure and is therefore hydrolysed.



PROTEIN SYNTHESIS

tRNAs

The accuracy of tRNA synthetases depends on a proofreading mechanism.

The tRNA binds to the enzyme by a two-step process:

1. the tRNA associates rapidly and dissociates slowly
2. the corrected tRNA triggers a conformational change that stabilises binding to the enzyme.

This mode of control is referred to as **KINETIC BOWL CORRECTION**.

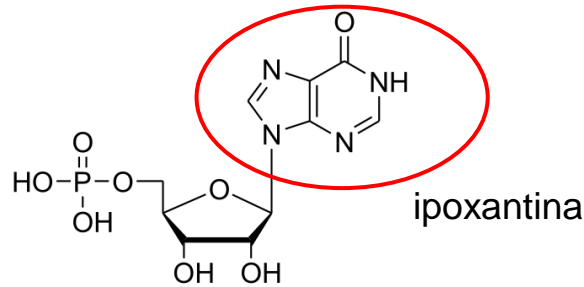


PROTEIN SYNTHESIS

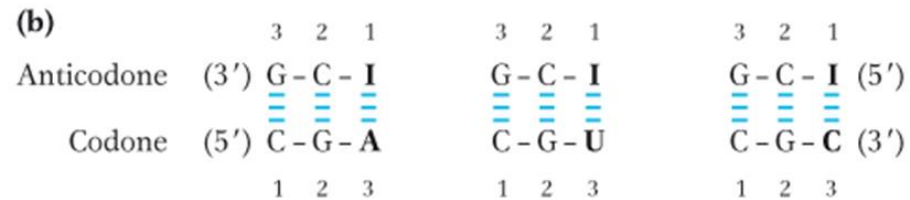
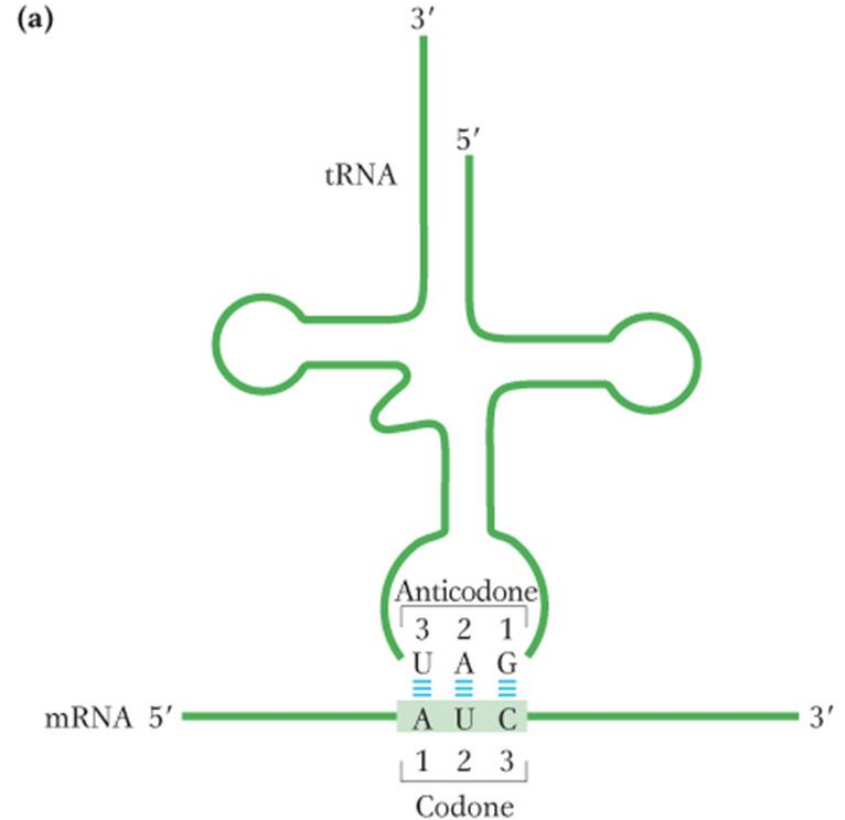
Genetic Code

the codon/anticodon pairing is described as **antiparallel**.

The first nucleotide on the anticodon can be an inosinate (I). This unique base allows for flexible pairing (**Wobble base**), enabling it to match with different nucleotides in the third position of the codon (U, C and A).



Inosine monophosphate





PROTEIN SYNTHESIS

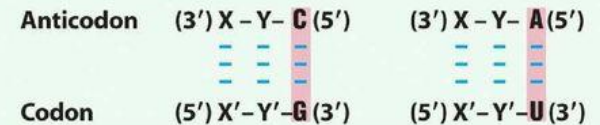
Genetic Code

1 ^a posizione	2 ^a posizione				3 ^a posizione
5'	U	C	A	G	3'
U	UUU Phe	UCU Ser	UAU Tyr	UGU Cys	U C A G
	UUC	UCC	UAC	UGC	
	UUA Leu	UCA	UAA Stop	UGA Stop	
	UUG	UCG	UAG Stop	UGG Trp	
C	CUU Leu	CCU Pro	CAU His	CGU Arg	U C A G
	CUC	CCC	CAC	CGC	
	CUA	CCA	CAA Gln	CGA	
	CUG	CCG	CAG	CGG	
A	AUU Ile	ACU Thr	AAU Asn	AGU Ser	U C A G
	AUC	ACC	AAC	AGC	
	AUA	ACA	AAA Lys	AGA Arg	
	AUG Met	ACG	AAG	AGG	
G	GUU Val	GCU Ala	GAU Asp	GGU Gly	U C A G
	GUC	GCC	GAC	GGC	
	GUA	GCA	GAA Glu	GGA	
	GUG	GCG	GAG	GGG	

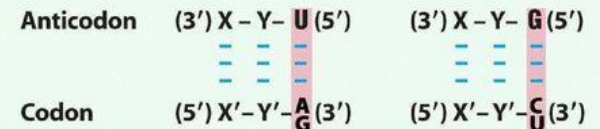
To translate the 61 codons, 32 tRNAs are needed- The codon's third position (or the anticodon's first position) is called the **wobble position**, where non-canonical base pairing can occur.

TABLE 27-4 How the Wobble Base of the Anticodon Determines the Number of Codons a tRNA Can Recognize

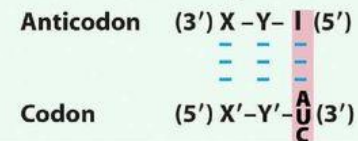
1. One codon recognized:



2. Two codons recognized:



3. Three codons recognized:



Note: X and Y denote bases complementary to and capable of strong Watson-Crick base pairing with X' and Y', respectively. Wobble bases—in the 3' position of codons and 5' position of anticodons—are shaded in pink.

The anticodon wobble base defines how many codons can be recognized from the same tRNA.

- C; A** → 1 codon
- U; G** → 2 codons
- I** → 3 codons



PROTEIN SYNTHESIS

Genetic Code

The first two bases of the codon very often determine the type of aa. In fact, if we examine codons coding for the same aa, they often differ only in the third base. This third base corresponds to the first base on the anticodon.

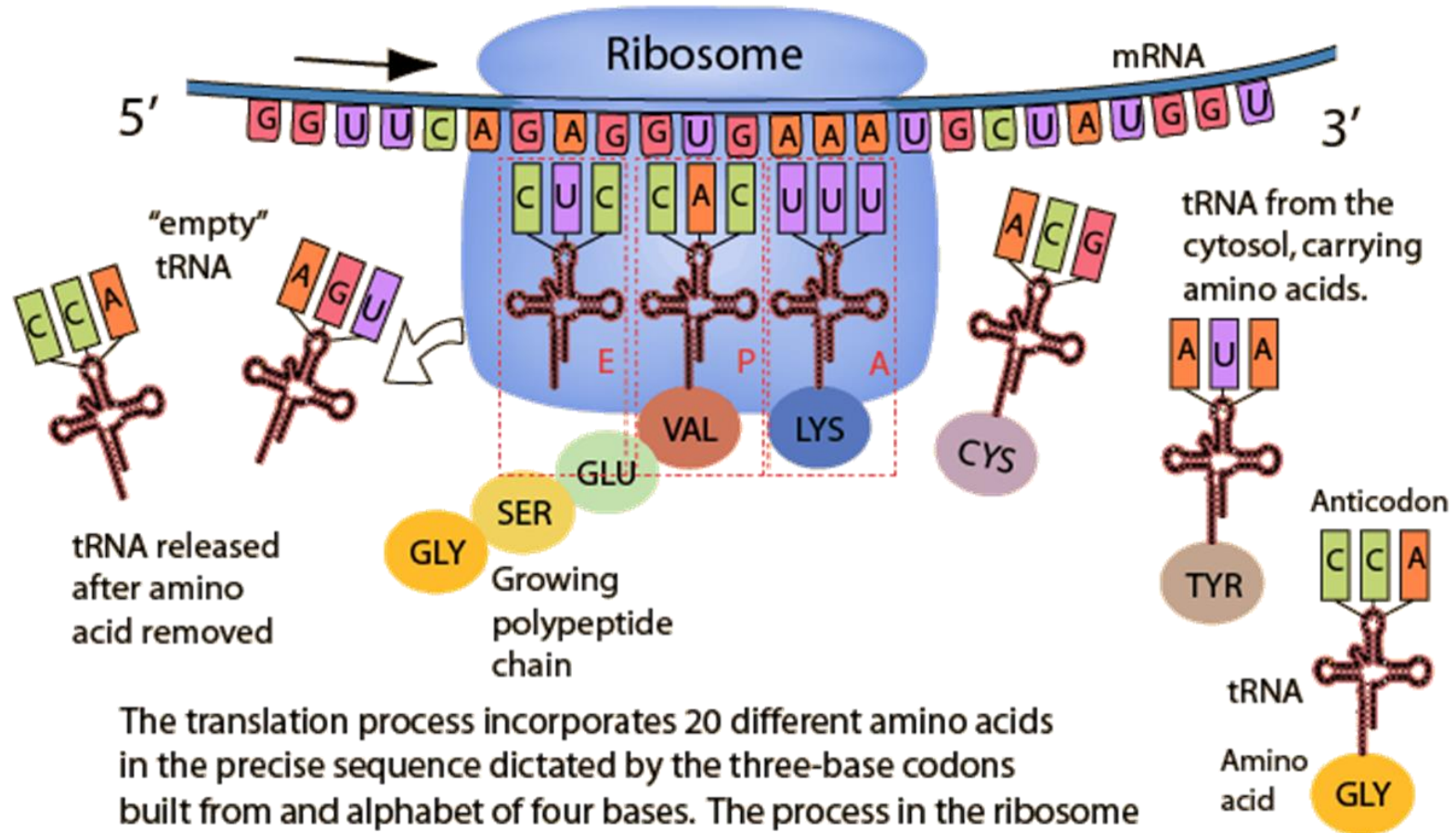
1 ^a posizione		2 ^a posizione				3 ^a posizione		
5'	U	C	A	G			3'	
U	UUU	Phe	UCU	Ser	UAU	Tyr	UGU	Cys
	UUC		UCC		UAC		UGC	
	UUA	Leu	UCA		UAA	Stop	UGA	Stop
	UUG		UCG		UAG	Stop	UGG	Trp
C	CUU	Leu	CCU	Pro	CAU	His	CGU	Arg
	CUC		CCC		CAC		CGC	
	CUA		CCA		CAA	Gln	CGA	
	CUG		CCG		CAG		CGG	
A	AUU	Ile	ACU	Thr	AAU	Asn	AGU	Ser
	AUC		ACC		AAC		AGC	
	AUA		ACA		AAA	Lys	AGA	
	AUG		ACG		AAG		AGG	
G	GUU	Val	GCU	Ala	GAU	Asp	GGU	Gly
	GUC		GCC		GAC		GGC	
	GUA		GCA		GAA	Glu	GGA	
	GUG		GCG		GAG		GGG	

- The first two bases of the codon form normal (canonical) H-links with the 2nd and 3rd bases of the anticodon
- Less stringent rules apply to the remaining position, so non-canonical pairings can occur
- The wobble pairment induces a faster dissociation of tRNA from mRNA

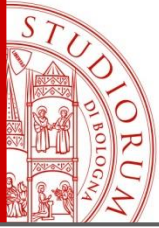
PROTEIN SYNTHESIS

Translation

It occurs in the cytosol in all cell types



The translation process incorporates 20 different amino acids in the precise sequence dictated by the three-base codons built from an alphabet of four bases. The process in the ribosome builds the polypeptide chains that will become proteins.



PROTEIN SYNTHESIS

Translation

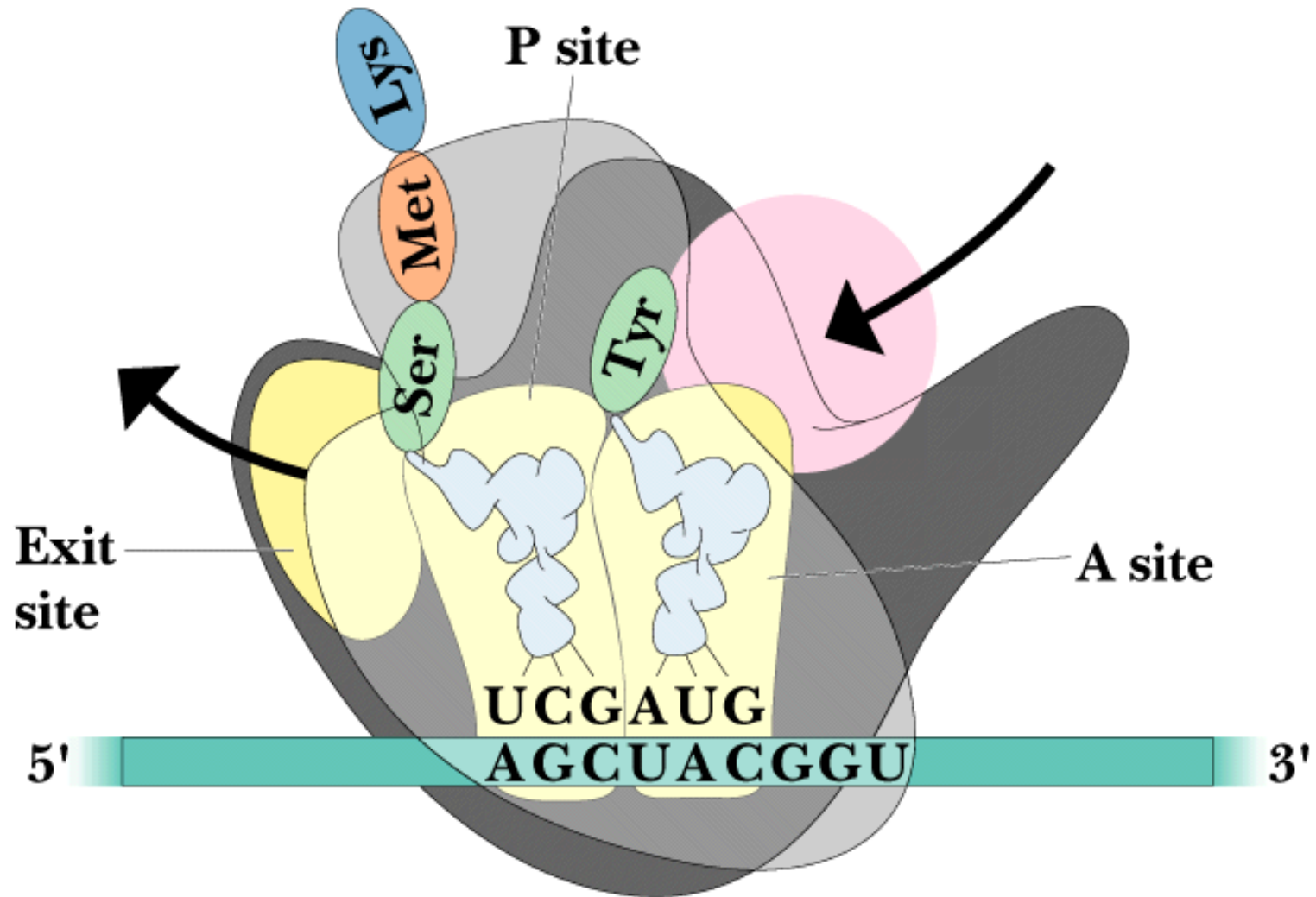
3 stages:

- **Initiation:** involves the binding of mRNA and an initiator aminoacyl-tRNA to the minor subunit of a ribosome, followed by the binding of the major subunit
- **Elongation:** synthesis of all peptide bonds with tRNA bound to the acceptor (A) and peptidyl (P) sites.
- **Termination:** occurs when the 'stop codon' is reached.



PROTEIN SYNTHESIS

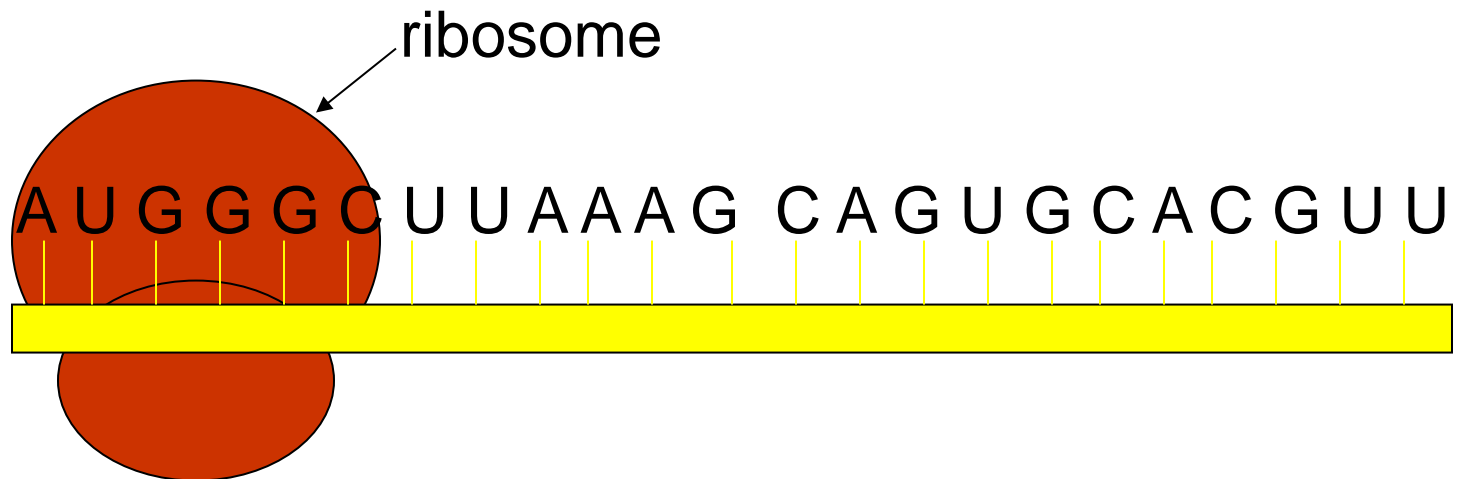
Translation



PROTEIN SYNTHESIS

Translation

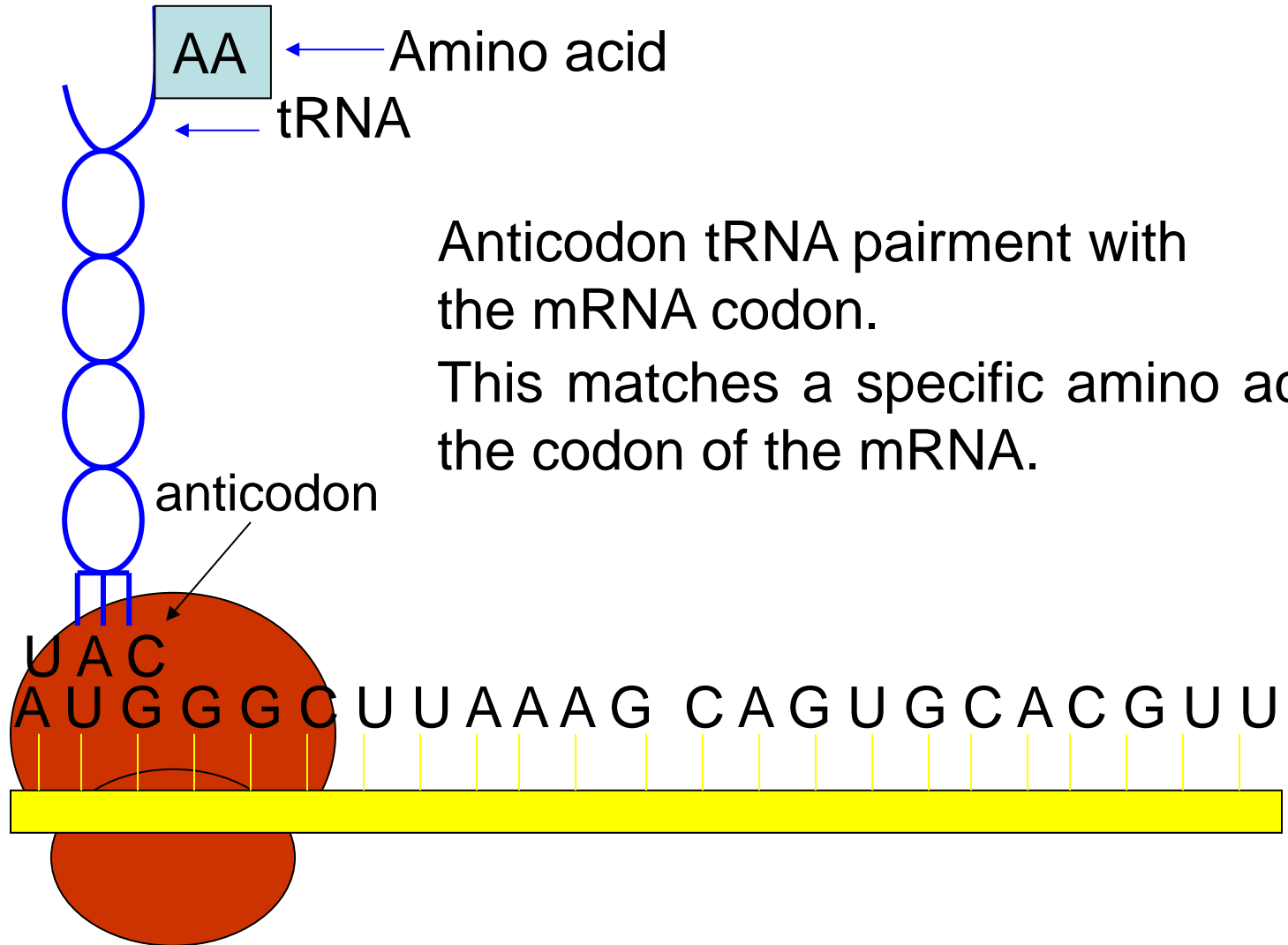
A ribosome interacts just with one mRNA

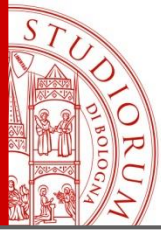




PROTEIN SYNTHESIS

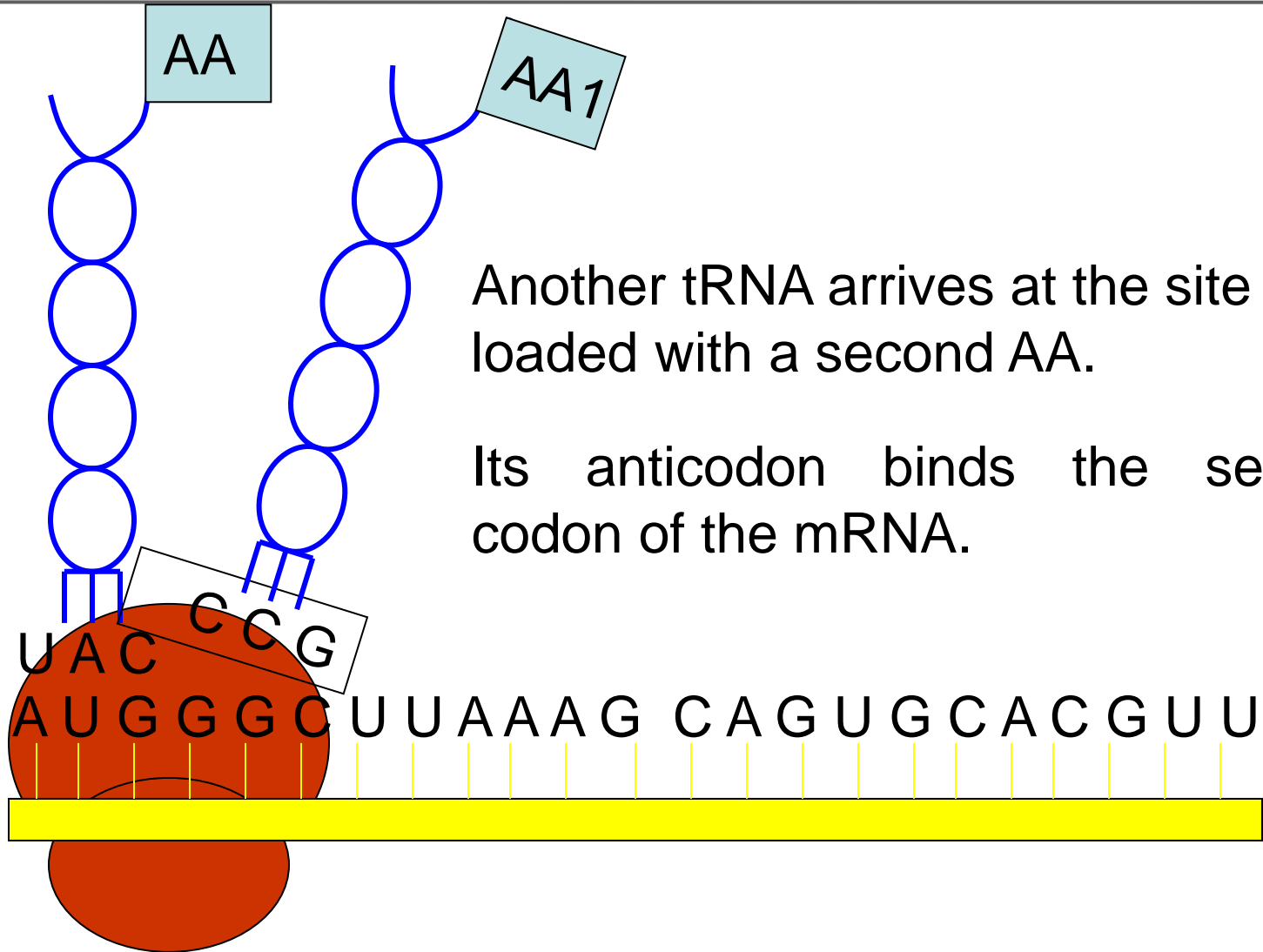
Translation





PROTEIN SYNTHESIS

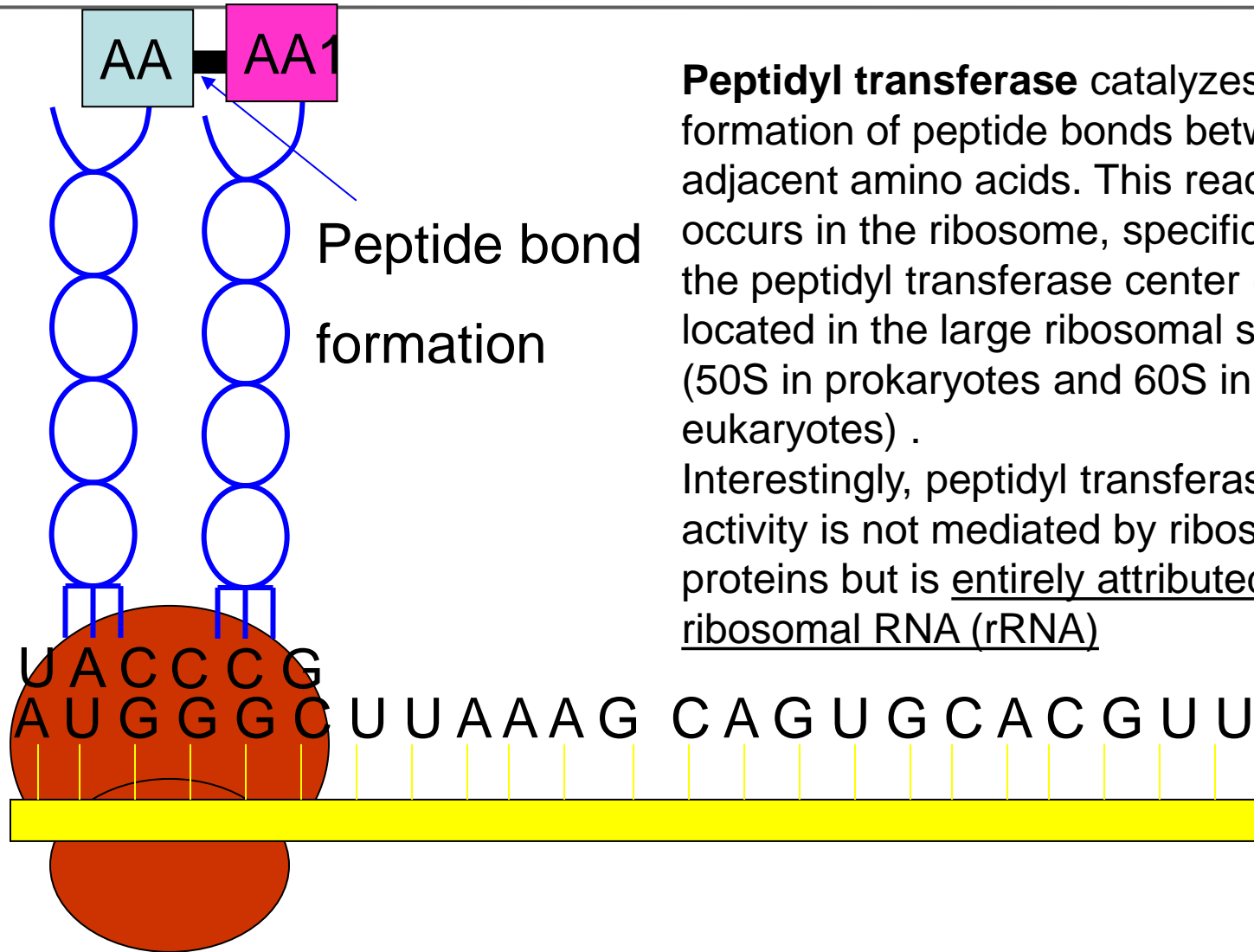
Translation





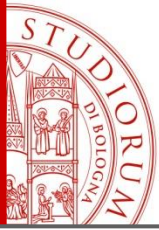
PROTEIN SYNTHESIS

Translation



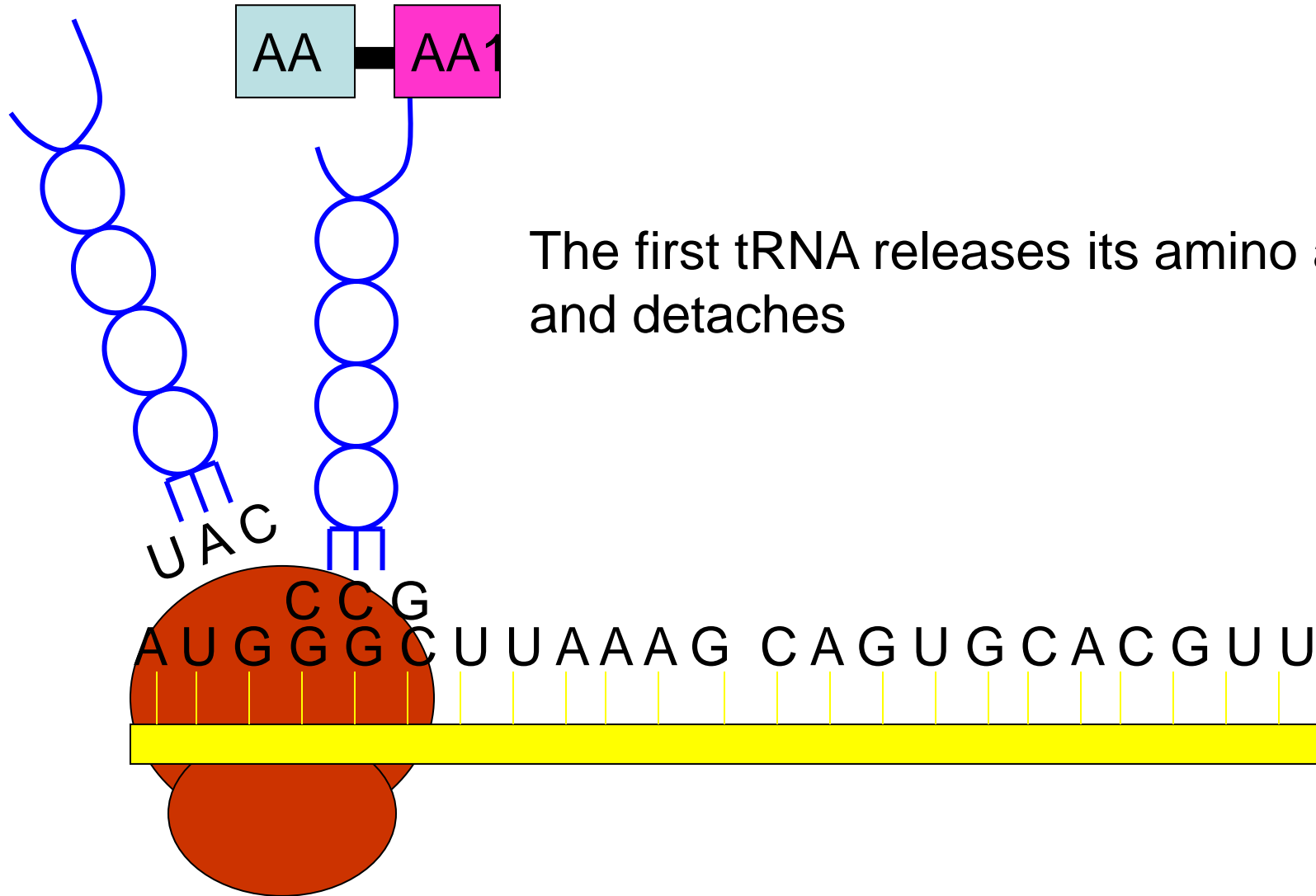
Peptide bond formation

Peptidyl transferase catalyzes the formation of peptide bonds between adjacent amino acids. This reaction occurs in the ribosome, specifically at the peptidyl transferase center (PTC) located in the large ribosomal subunit (50S in prokaryotes and 60S in eukaryotes) . Interestingly, peptidyl transferase activity is not mediated by ribosomal proteins but is entirely attributed to ribosomal RNA (rRNA)

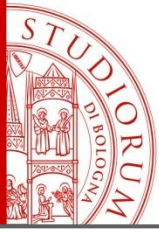


PROTEIN SYNTHESIS

Translation

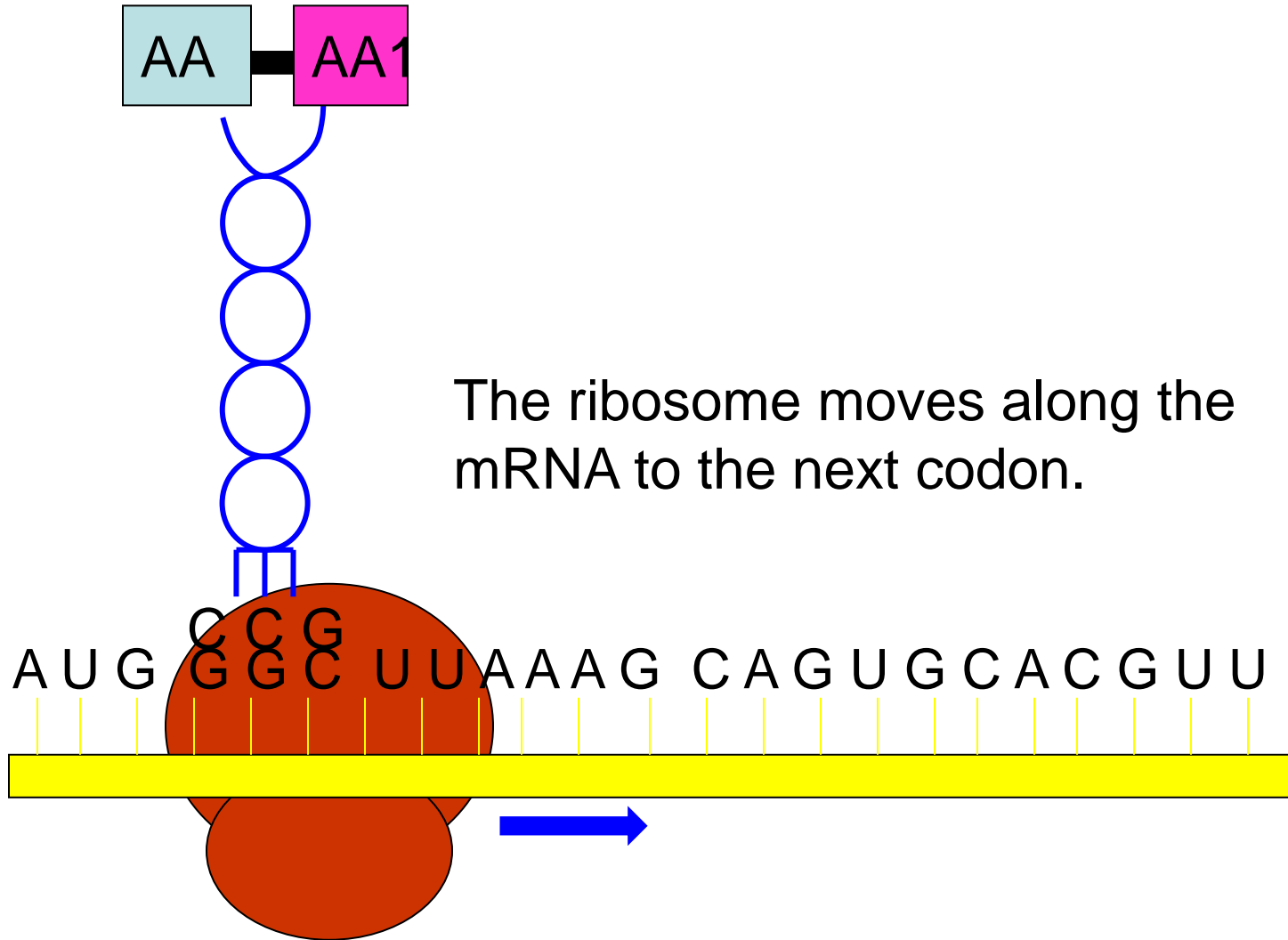


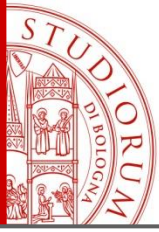
The first tRNA releases its amino acid and detaches



PROTEIN SYNTHESIS

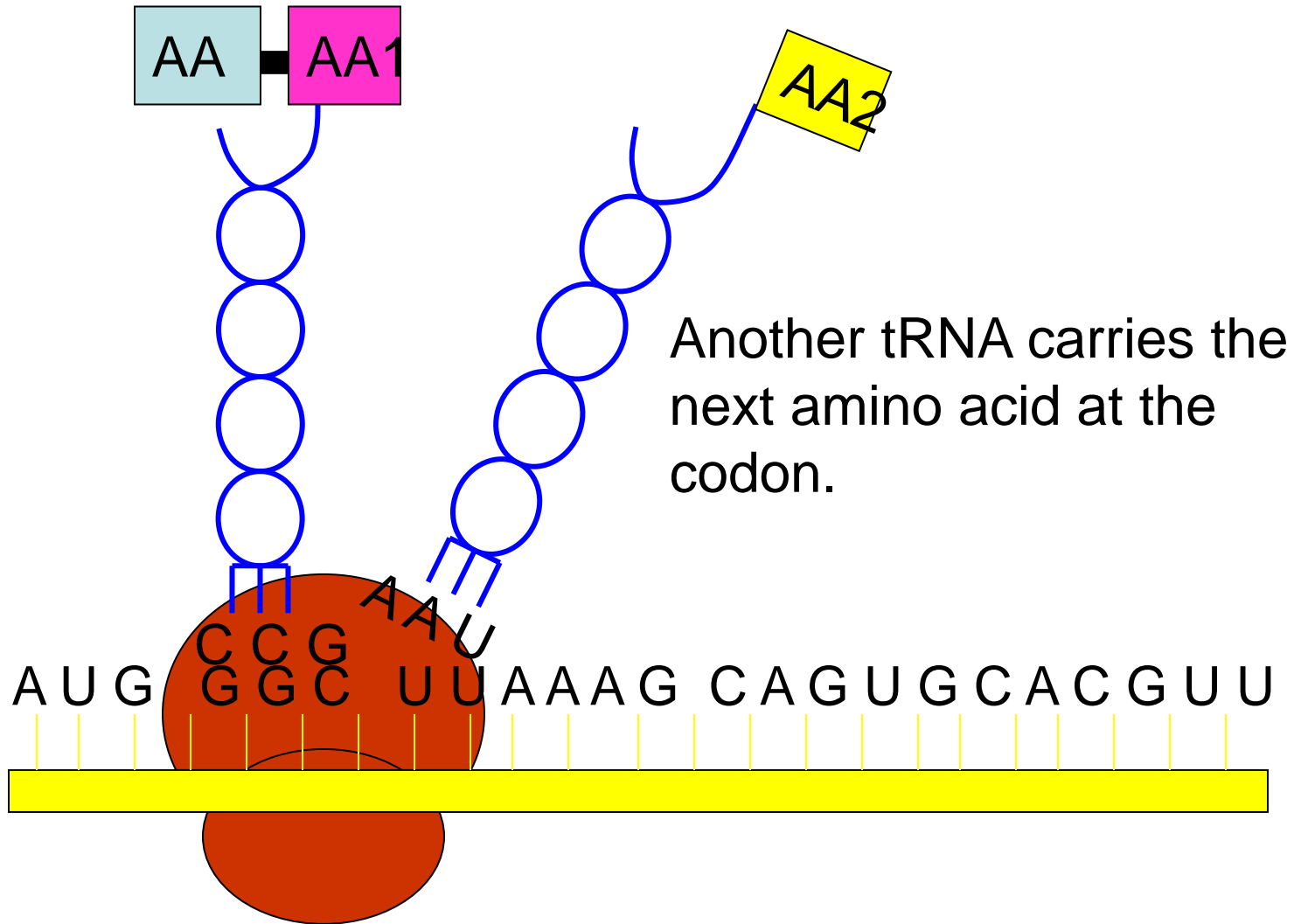
Translation





PROTEIN SYNTHESIS

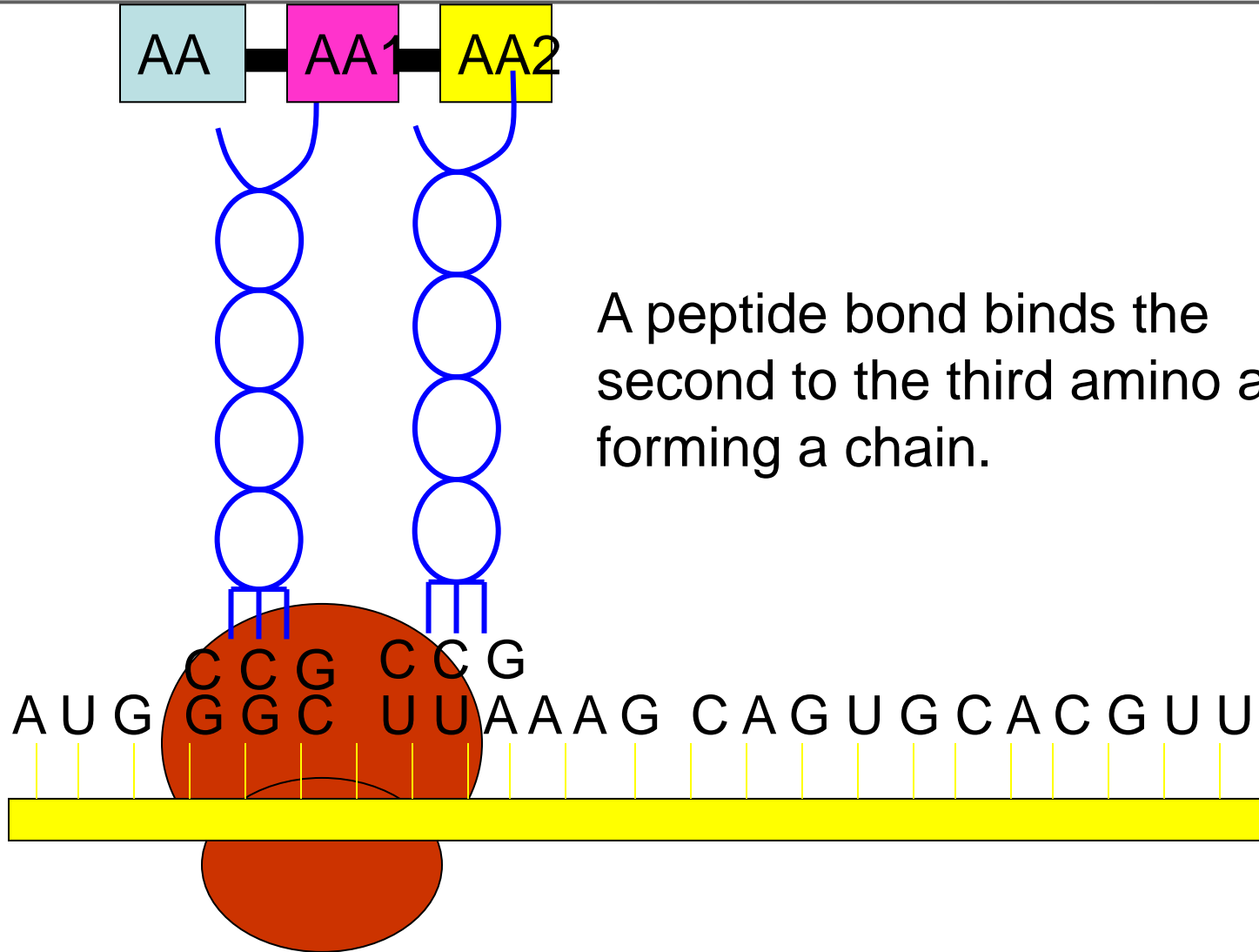
Translation





PROTEIN SYNTHESIS

Translation

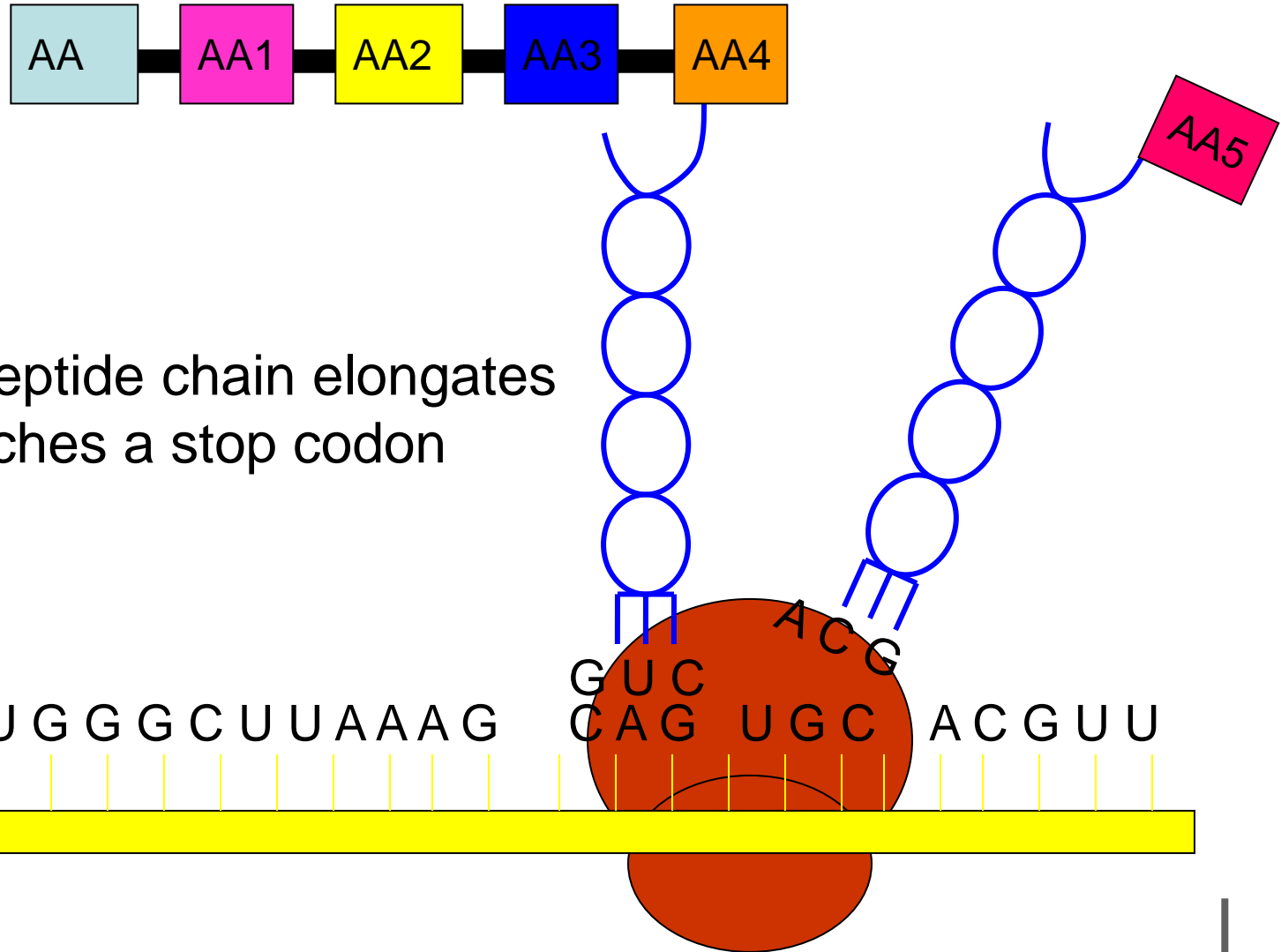


A peptide bond binds the second to the third amino acid, forming a chain.



PROTEIN SYNTHESIS

Translation





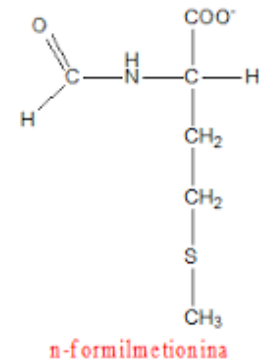
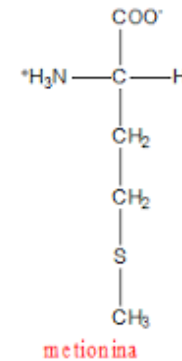
PROTEIN SYNTHESIS

Initiation

The starting codon is AUG (Two different tRNAs: one if at the start position, one coding if for an aa inside the chain).

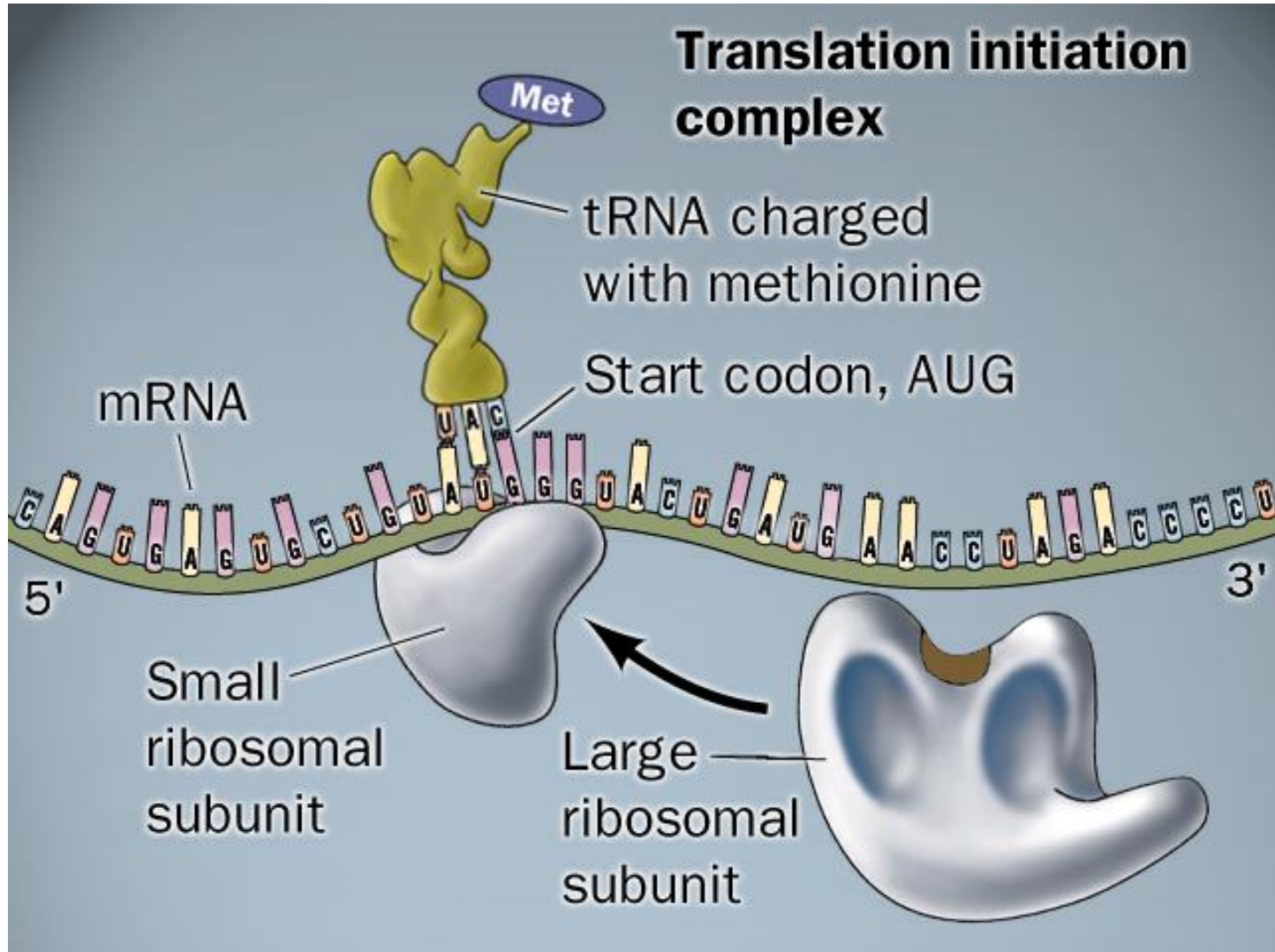
In bacteria, the AUG triplet encodes an N-formyl-methionine if present as the start codon, or methionine if inside the chain. N-formyl methionine is the first aa of all E. coli proteins, but is cut off in about half of the proteins.

In eukaryotes it always encodes normal methionine.



PROTEIN SYNTHESIS

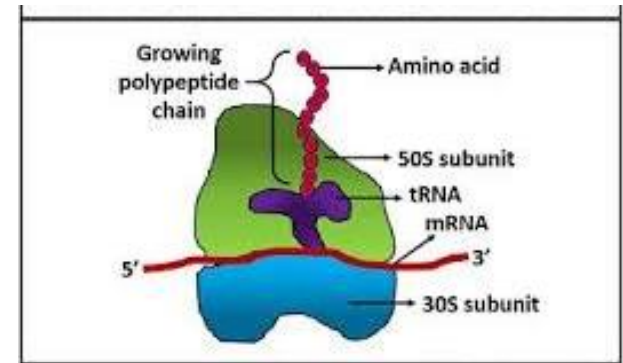
Initiation



PROTEIN SYNTHESIS

Initiation

- It proceeds from the N-terminal to C-terminal end;
- elongation occurs by binding the growing polypeptide to the AA residue on the incoming t-RNA.
- Ribosomes read mRNA in the 5'-3' direction



Key Components in prokaryotes

mRNA: The start codon is typically AUG, which codes for N-formylmethionine (fMet) as the first amino acid.

• Ribosomal Subunits: small subunit (30S) and a large subunit (50S), which together form a functional 70S ribosome during translation.

• Initiation Factors (IFs): IF-1, IF-2, and IF-3—are essential for the assembly of the initiation complex.

• Initiator tRNA: The initiator tRNA carries fMet and is crucial for starting the translation process.

PROTEIN SYNTHESIS

Prokaryotes

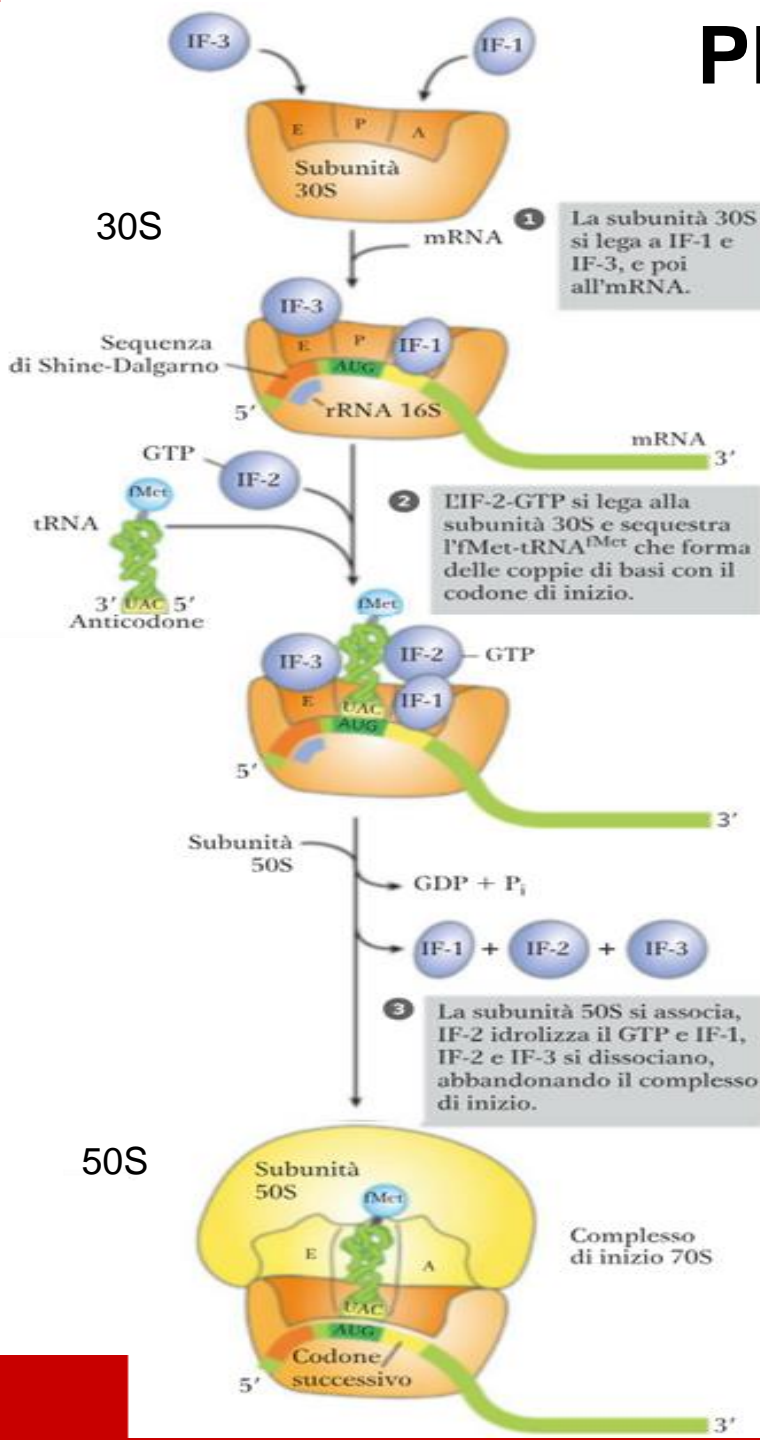
INITIATION

-In 16s rRNA, *Shine-Dalgarno sequence* recognition placed before the start codon (-8 nt), consensus sequence AGGAGG in mRNA. This is the binding site of mRNA into 30S ribosome.

- The initiation protein factors (IF-1, IF-3), 30S, mRNA, IF-2-GTP, N-formyl-Met-tRNA, form the 30S initiation complex.

- The complex recruits the 50S subunit

A: aminoacyl site; P: peptidic side E: exit site

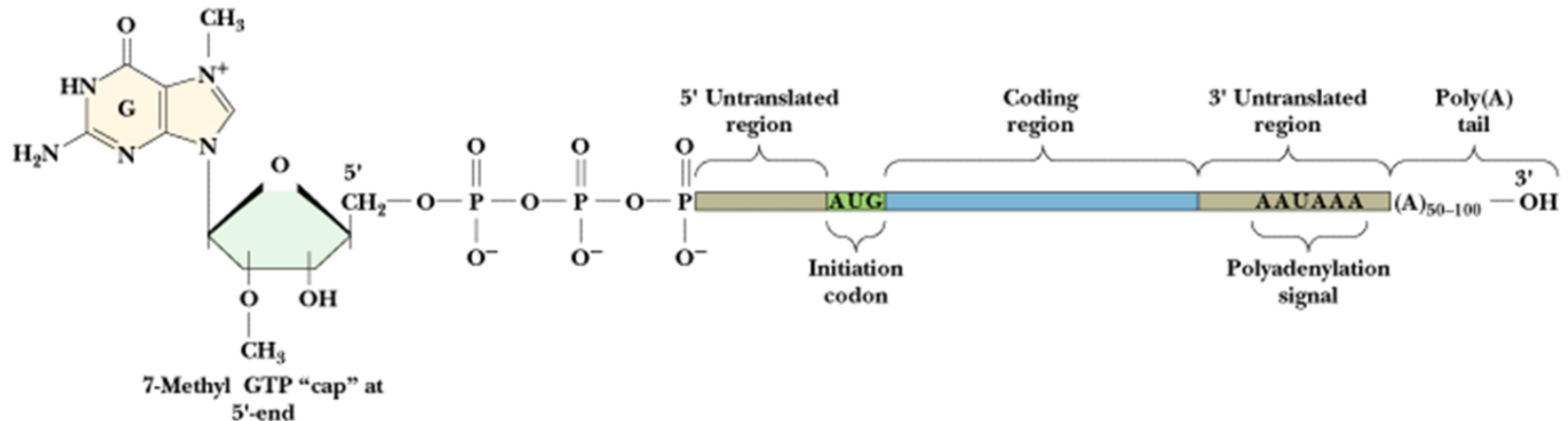




PROTEIN SYNTHESIS

Eukaryotes

- It begins with the formation of a ternary complex of the eukaryotic initiator factor (eIF)-2, GTP and tRNA linked with Met (MetRNA_t)
- This complex binds the 40S rRNA in the P site thanks to the enter of eIF3 and eIF1A, thus forming the Preinitiation Complex
- This complex recognizes the eIF-4E and eIF4G already linked with the cap in 5' end and the eIF4G to the poly(A) at the 3' end of mRNA
- 40S rRNA slides in mRNA until the MetRNA_t recognizes the start codon in mRNA.
- GTP is hydrolized, eIFs dissociate, the 60s rRNA joins the complex and the translation begins.



PROTEIN SYNTHESIS

Eukaryotes

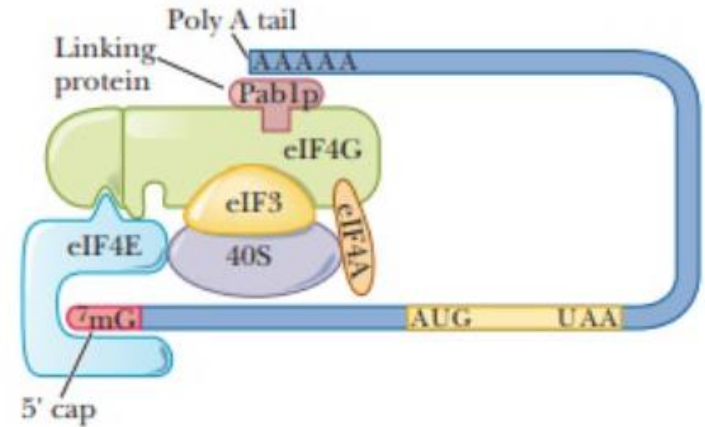
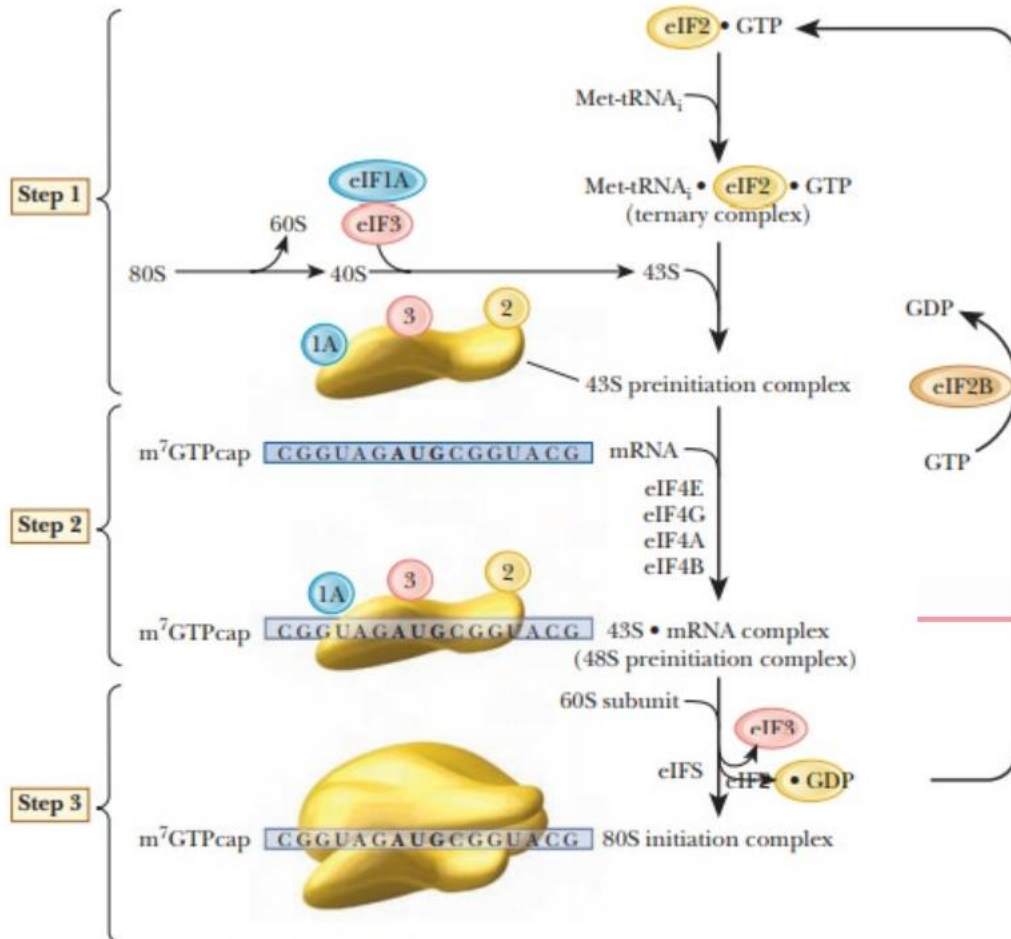
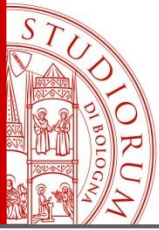


FIGURE 12.21 mRNA looping in eukaryotic translation initiation. Initiation factor eIF4G serves as a multipurpose adapter to engage the ⁷methyl-G cap:eIF4E complex, the Pab1p:poly(A) tract, and the 40S ribosomal subunit in eukaryotic translation initiation. (Adapted with permission from Heutze, H. W., 1997. eIF4G: A multipurpose ribosome adapter? *Science* 275, 500–501. Copyright © 1997 AAAS.)



PROTEIN SYNTHESIS

Elongation

1. Second tRNA-AA binding
2. Peptide bond formation
3. tRNA translocation



PROTEIN SYNTHESIS

Elongation

- Elongation factors (EF) are fundamental for cell functions so are highly abundant in the cytosol (in E. Coli EF-Tu represents the 5% of total proteins)
- EF-Tu binds the aminoacyl-tRNA and GTP
- the aminoacyl-tRNA binds the A site of the small subunit of rRNA as a complex with 2 EF-Tu and 2 GTP
- GTP is hydrolyzed and the complex EF-Tu:GDP dissociates
- EF-Tu is recycled thanks to a substitution from GDP to GTP

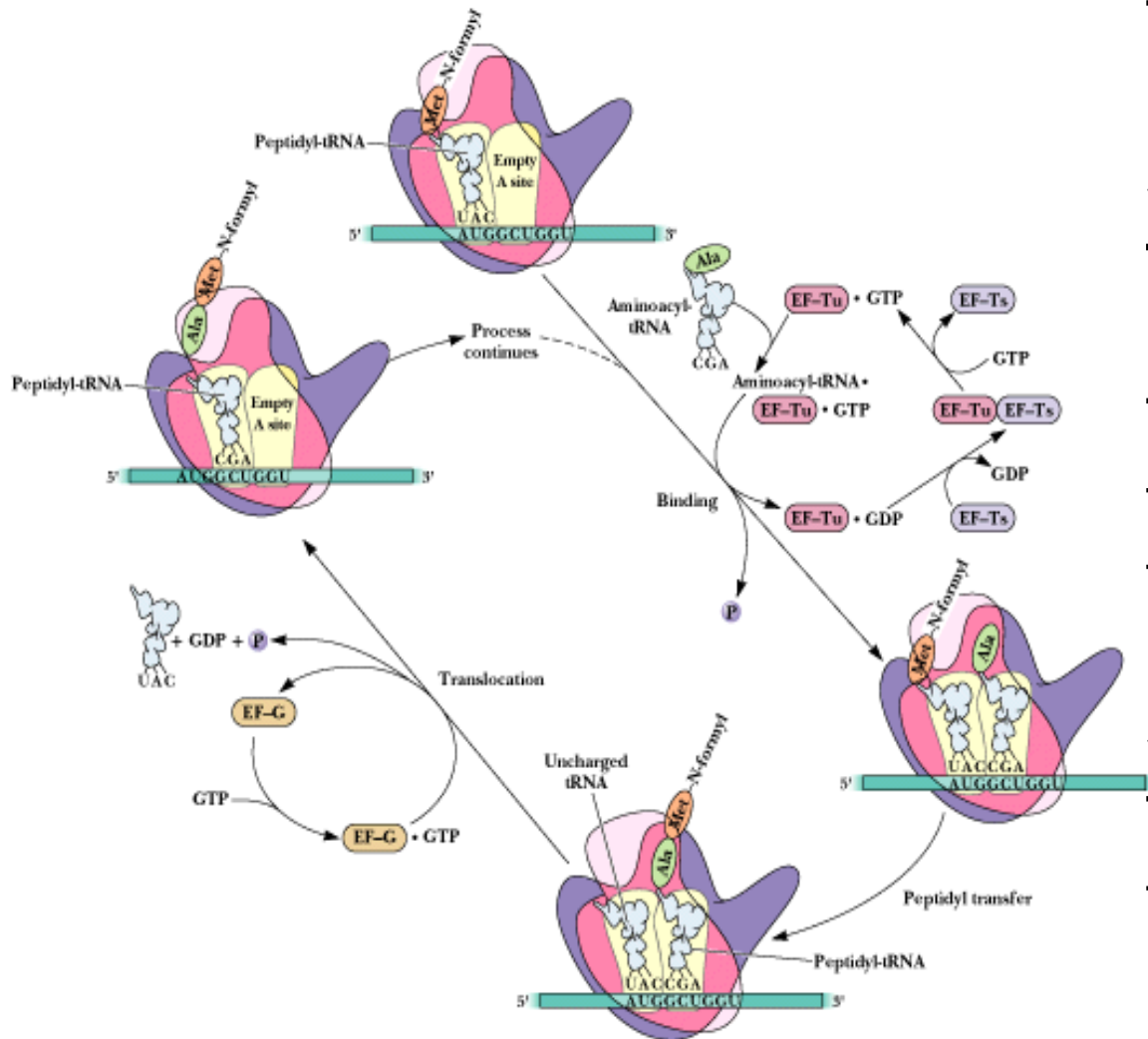
PROTEIN SYNTHESIS

Elongation

This is the central reaction in protein synthesis by **peptidyl transferase (23S rRNA in prokaryotes)**.

The 'reaction center' of the 23S rRNA is among the most highly conserved in all biological systems.

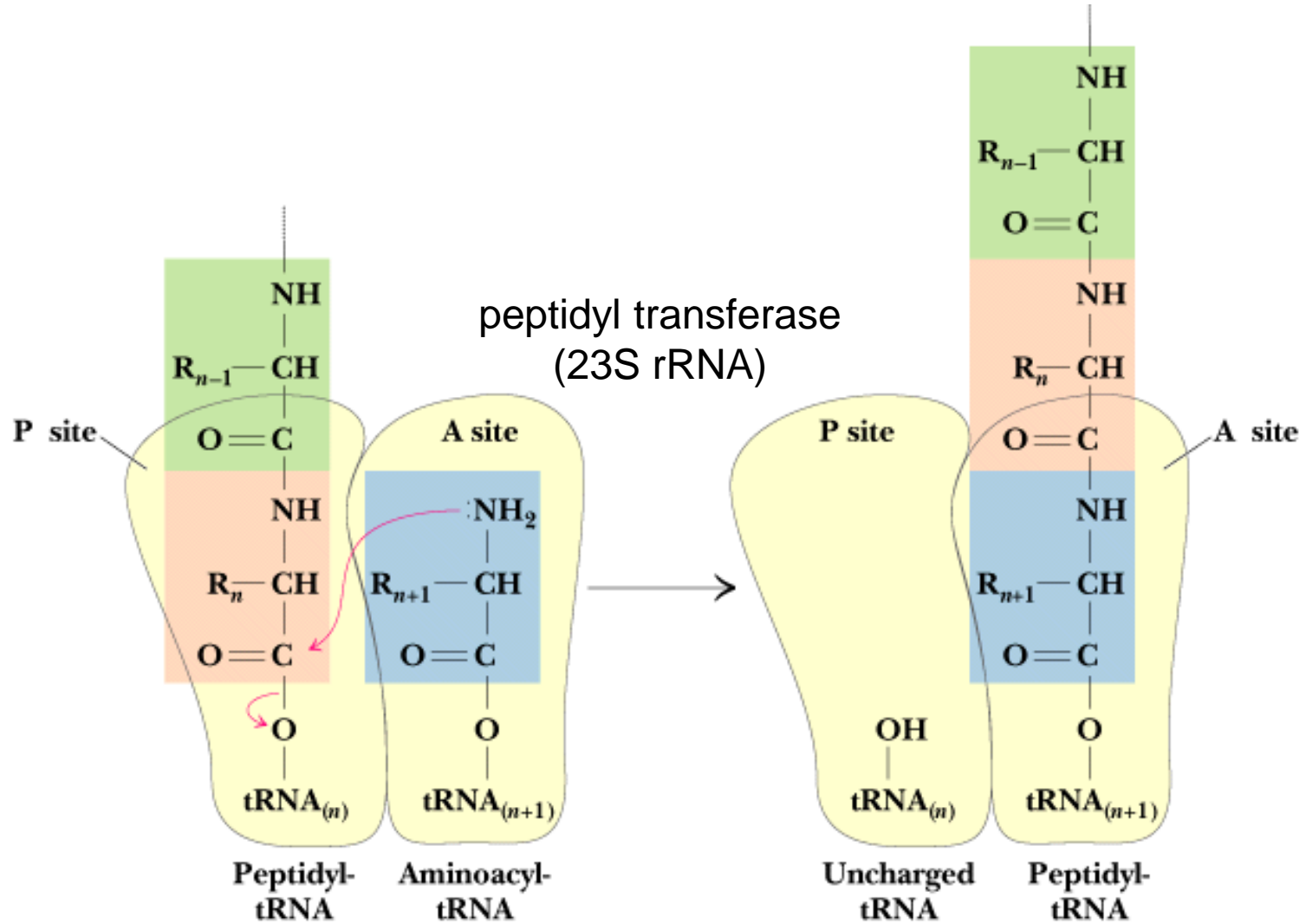
This is followed by the translocation of the peptidyl-tRNA from the A site to the P site.

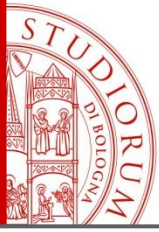




PROTEIN SYNTHESIS

Elongation





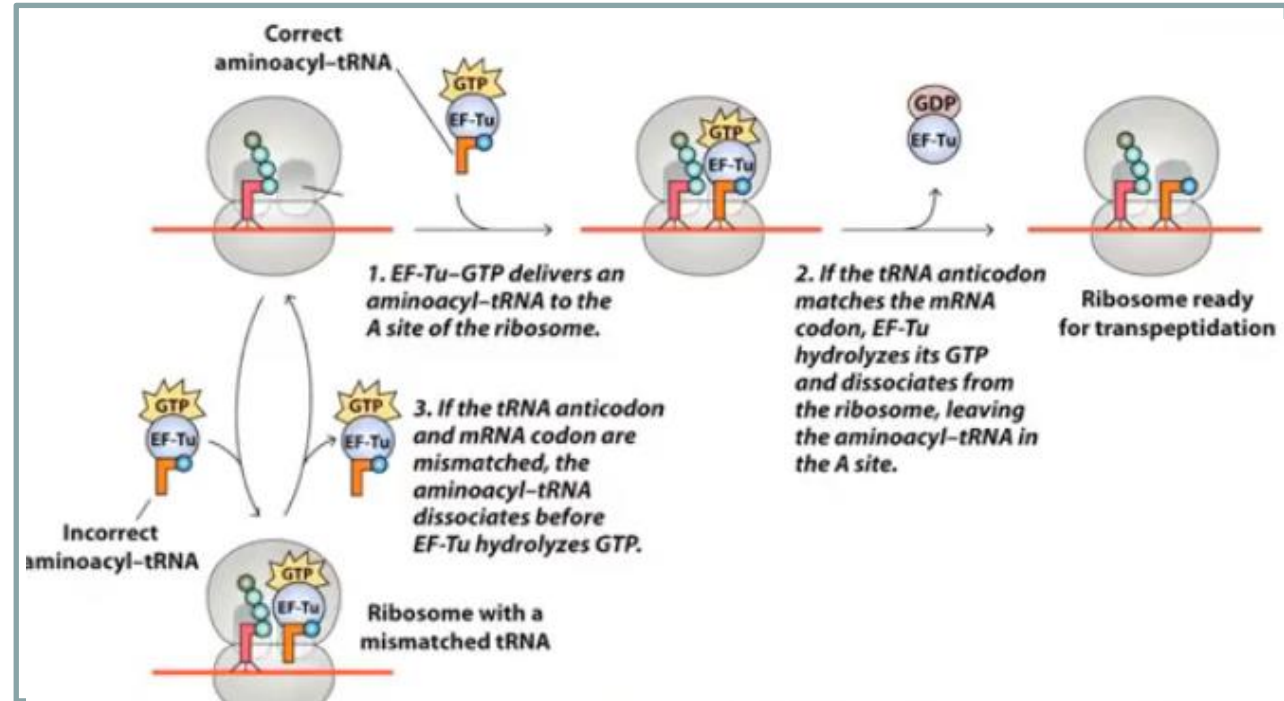
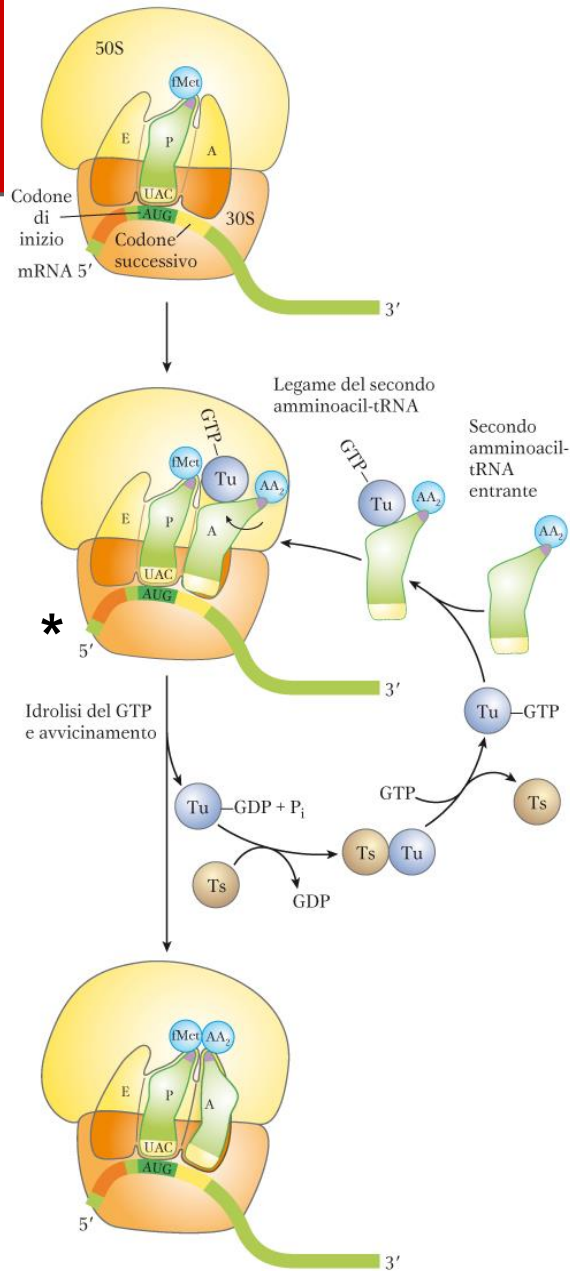
PROTEIN SYNTHESIS

Elongation

- Three GTPs are hydrolysed for each amino acid incorporated into the peptide.
- Hydrolysis essentially drives conformational changes.
- A total of five high-energy phosphoric bonds are spent per added amino acid residue - three GTPs here and two in amino acid activation by aminoacyl-tRNA synthesis.

PROTEIN SYNTHESIS

Elongation



* Before GTP hydrolysis given by EF-Tu, the aminoacyl-tRNA synthetase verifies the correct codon-anticodon pairing (*proofreading* activity) in the A site.

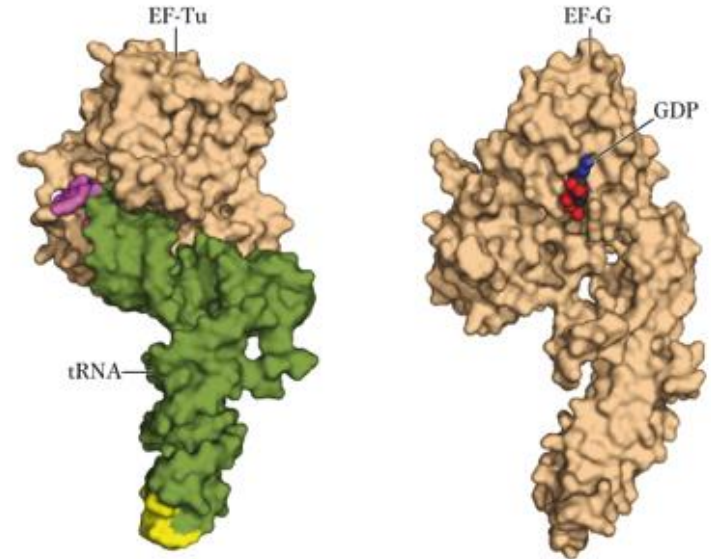
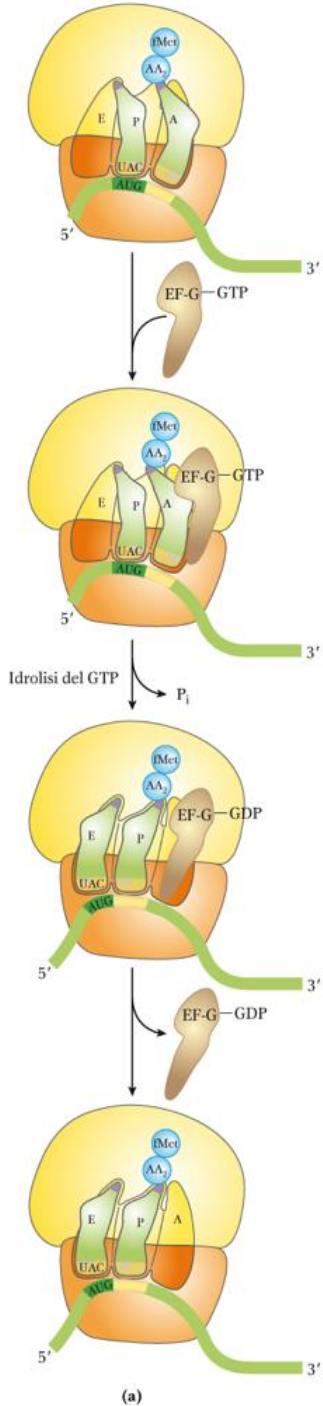
PROTEIN SYNTHESIS

Elongation - translocation

Procarvotytes:
EF-G -GTP

Eucaryotes:
eEF2-GTP

EF-G, a translocase, binds in the A site, and promotes the translocation hydrolyzing the GTP to GDP



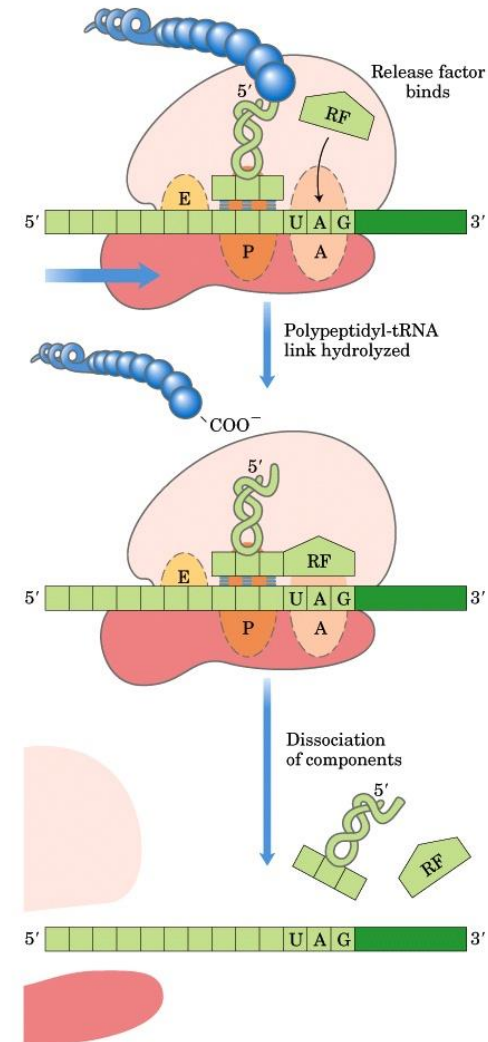
(b)

Structural analogy
between EF-G and
EF-Tu-tRNA

PROTEIN SYNTHESIS

Termination

- Proteins known as release factors recognise the stop codon at the A site
- The presence of release factors with a nonsense codon at the A site induce the peptidyl transferase to a hydrolase activity, which cleaves the peptide chain from the tRNA carrying it.

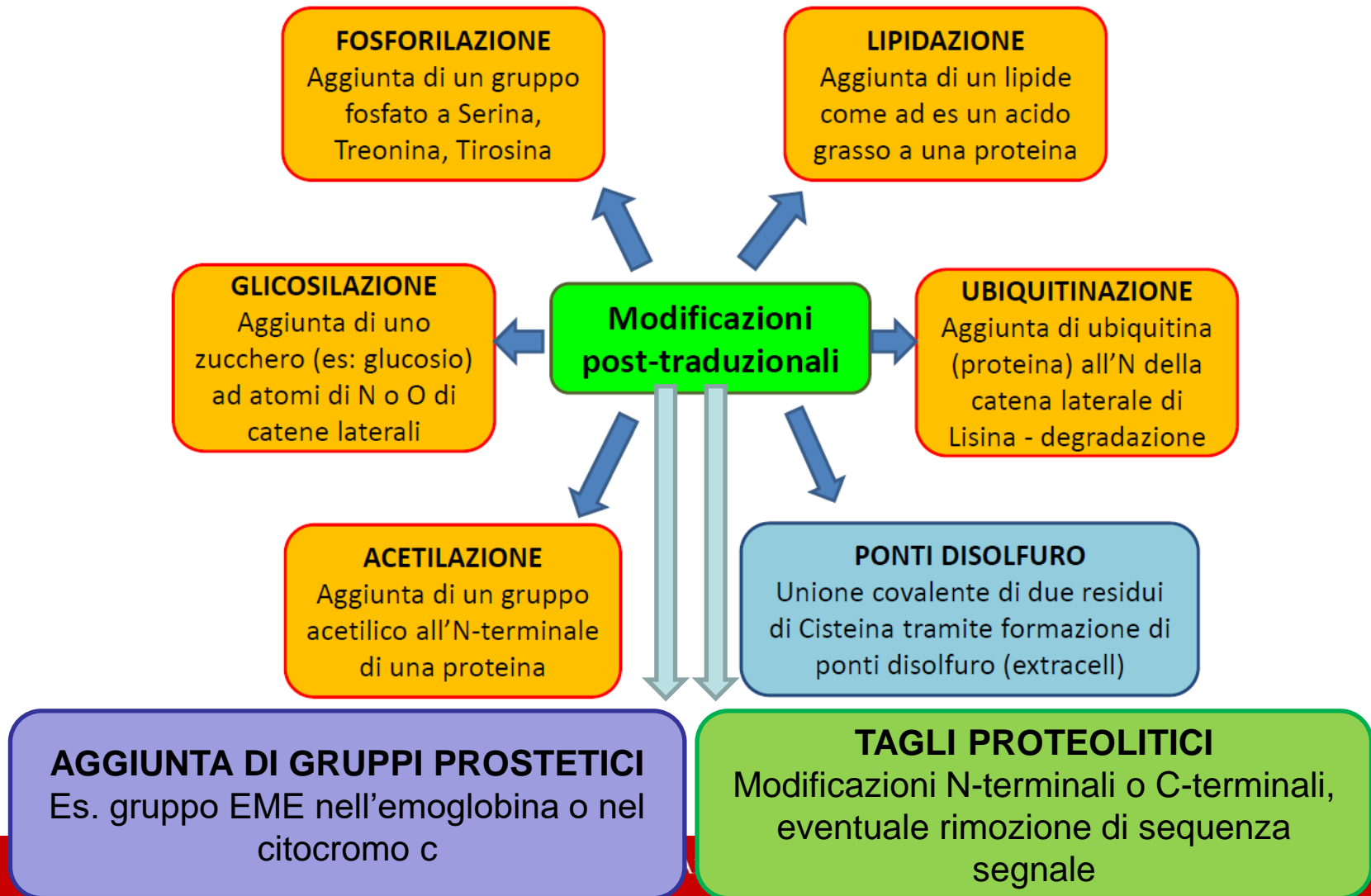




PROTEIN SYNTHESIS

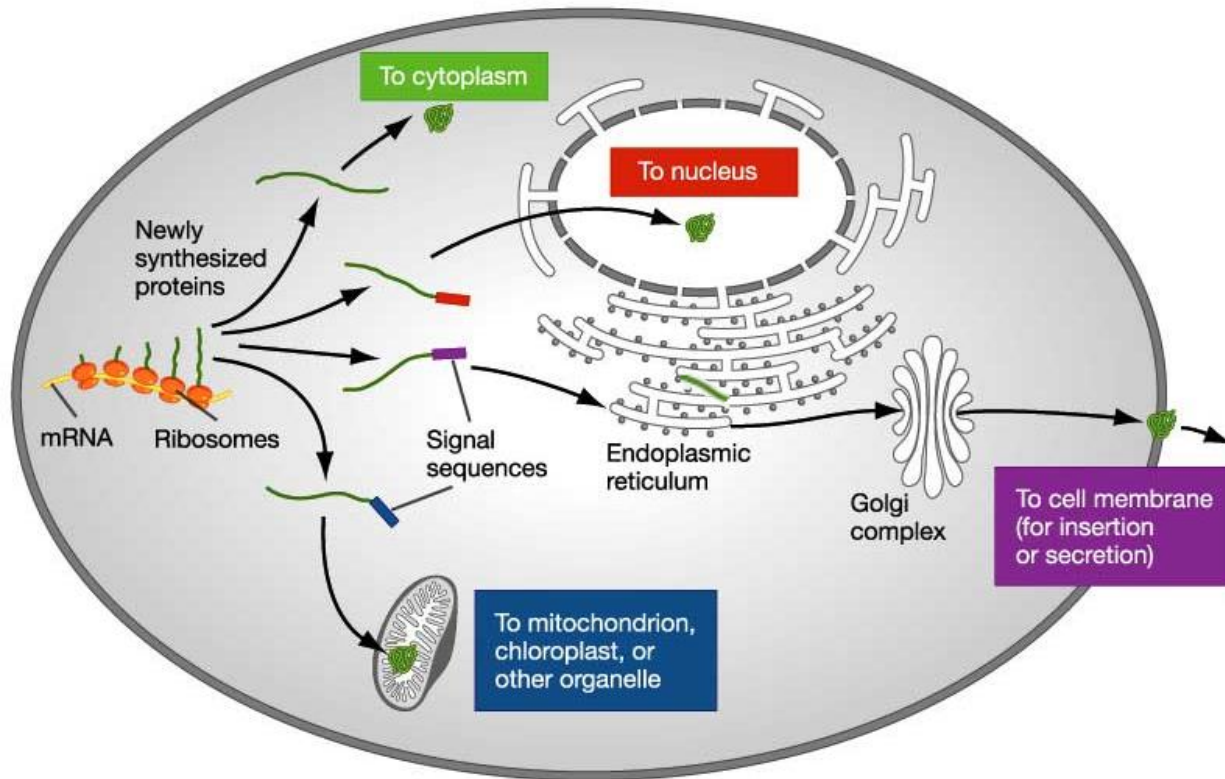
Post-translational modifications

Modificazioni post-traduzionali



SINTESI PROTEICA

Dopo la traduzione



An essential process for **membrane and secretory proteins**.

These proteins are synthesised with a 'peptide leader' sequence, also known as a 'signal sequence' of approximately 16-26 amino acids. The signal sequence directs the newly synthesised protein to its destination.

Kahoot!