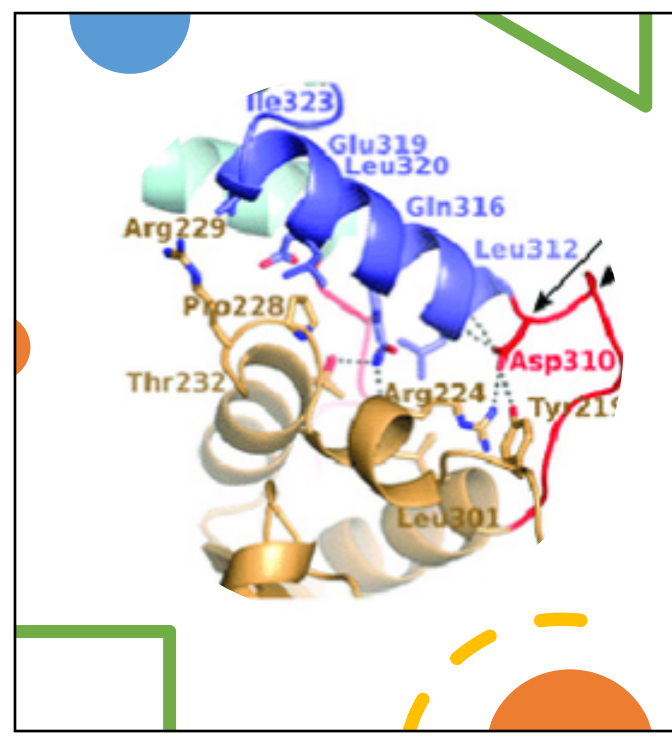

Lecture 1

Introduction to Molecular Biophysics

(Biophysics at molecule level)

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Department Of Applied Chemistry

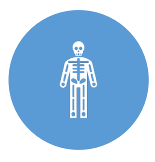
1

What does Biophysics study (at molecule level)?

It studies and explains the physical behaviour of life at the molecular level.

It uses the tools and terminology of physical chemistry to describe the dynamic of living organisms

It answers some question about the dynamic and the kinetics of the biomolecules



It gives a logic about the dynamics in the living molecules

2

Biophysics and proteins

- What does Biophysics (at at molecule level) study?
- Biophysics is the of study structural and functional biomolecules such as protein, DNA and RNA etc.
- Since, Proteins, DNA and RNA are the biomolecules,
- Biophysics can explain the Protein function and structure, using kinetics, thermodynamics and characterization facilities to understand the biological systems.

3

Biophysics of the biomolecules

- How do we study the biophysics of biomolecules ?
- There are several biochemical and biophysical techniques to study biomolecules.
- What are the techniques that are used to study the biophysics of proteins?

1. Genetic engineering. (described later)
2. Cloning techniques. (described later)
3. Protein expression and purification methods.
4. Structural and functional assays. Such as: Protein-protein interactions, protein-ligand interactions (for dug design) and DNA or RNA-protein interactions.

4

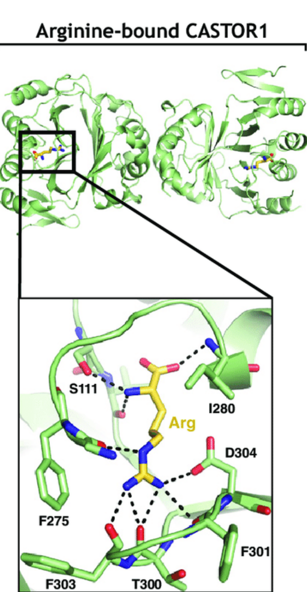
Techniques used to study the biophysics of at molecule level?

1. Expression of Genes in Bacteria, Yeast, and Cultured Mammalian Cells.
2. Protein purification Methods.
3. **Examination of protein purity**
4. Protein Crystallization for structure analysis.
5. Protein NMR
6. **Optical spectroscopic techniques for biomolecules. 1- Absorbance.**
7. **Optical spectroscopic techniques for biomolecules** 2- Fluorescence .
8. Kinetics of proteins.
9. Circular dichroism.
10. **Sedimentation assays**
11. Electron microscopy.
12. Thermodynamics of proteins.
13. Mass spectroscopy .

5

What is Structural biology?

- **Structural biology** is the study of the molecular **structure** and dynamics of **biological** macromolecules, particularly proteins and nucleic acids.
- It shows how alterations in their **structures** affect their function.
- Why it is useful?
- Because is explains how biological molecules are built (3D shape) and therefore, how it does work.
- What is that for?
- It is to understand the diseases formation and to design dugs for treatment.



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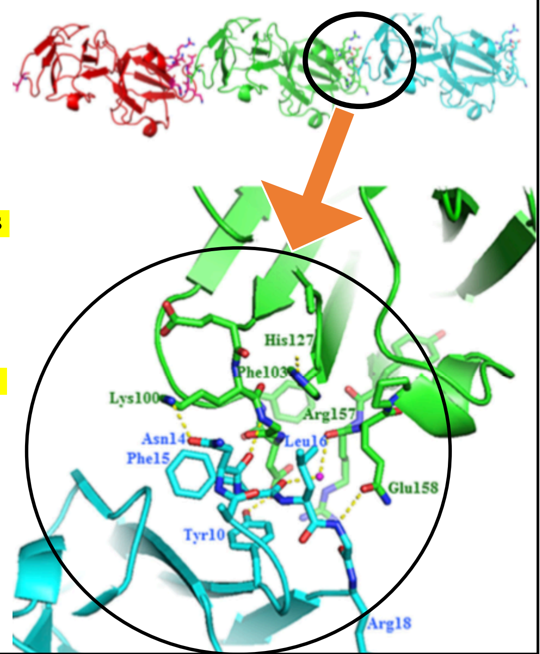
What are Protein-protein interactions ?



- **Protein–protein interactions** (PPIs) are physical contacts between two or more **protein** molecules.
- It is as a result of biochemical events driven by **interactions** that include electrostatic forces, hydrogen bonding and the hydrophobic effect.

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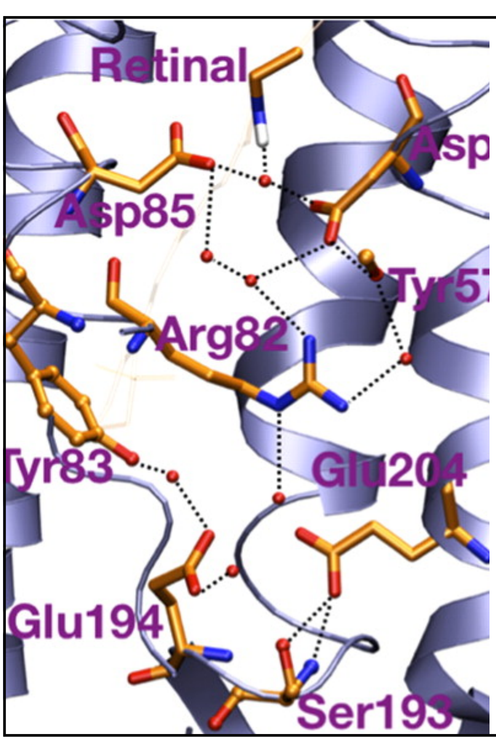


What are several types of bonds and forces that hold a protein and affect its interaction with other?

- **There are several types of bonds and forces that hold a protein in its tertiary structure.**

1. **Hydrophobic interactions** greatly contribute to the folding and shaping of a protein.
2. **Hydrogen bonding: between OH and H.**
3. **The ionic bonding** can occur between the positively and negatively charged "R" groups.
4. **The disulphide bridge**. Between S-S of 2 cysteine molecules.
5. **Van der Waals forces** stabilize protein structure.

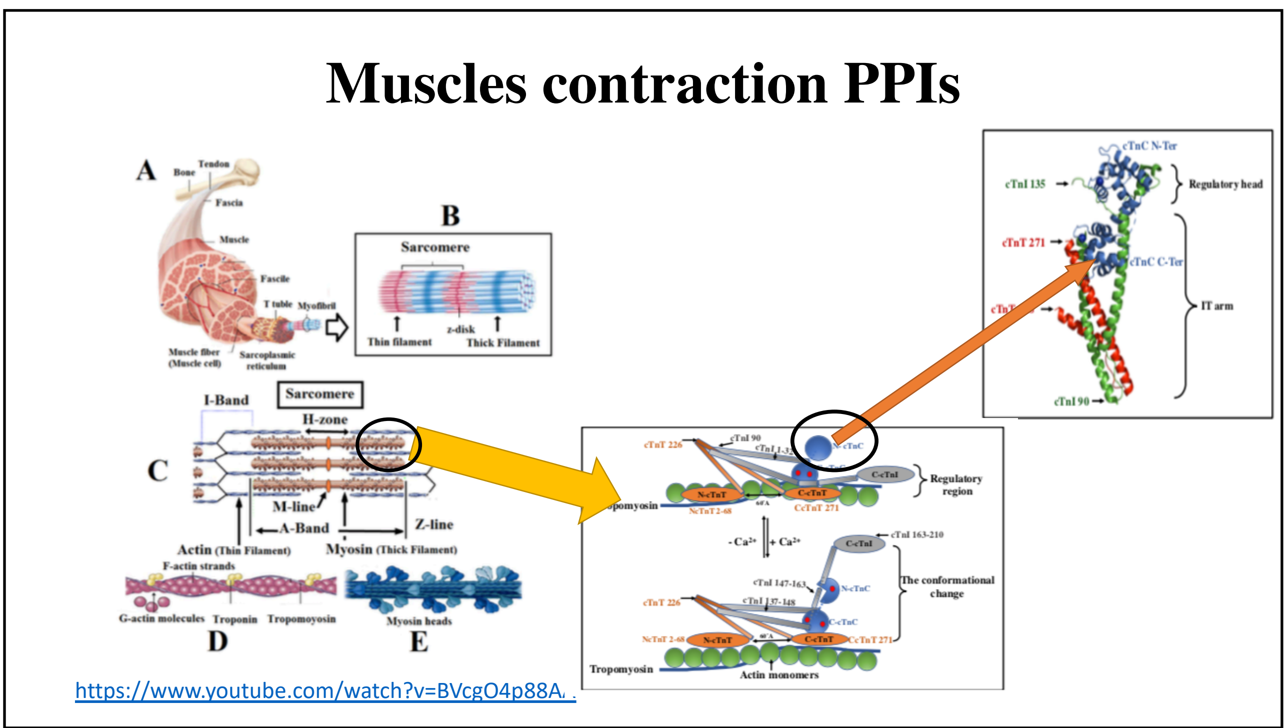
8

Examples:

- Muscles contraction (striated muscles) are regulated by **Protein–protein interactions** which result in a mechanical movement of the body. The same with (cardiac and smooth muscles) but for different functions.
- The activity of the cell is regulated by extracellular signals. Signal propagation inside and/or along the interior of cells depends on PPIs between the various signalling molecules.
- Membrane proteins are carried by PPIs.
- In cell metabolism, many biosynthetic processes enzymes interact with each other to produce small compounds or other macromolecules.

9

Muscles contraction PPIs



https://www.youtube.com/watch?v=BVcgO4p88A

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Example of function measured by biophysics

e.g: We measure the difference on the contractility/ time between a healthy heart (WT) and a diseased heart (mutation).

Normal heart

Heart with left ventricular hypertrophy

EF LS

S1 uM

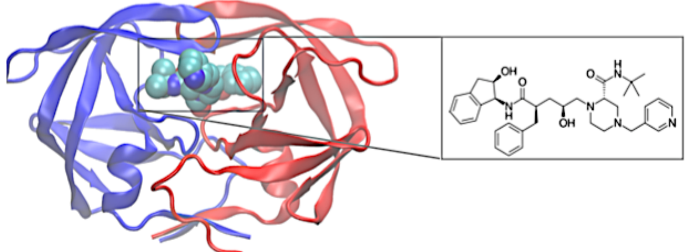
WT

Mutation

11

What are protein-ligand interactions?

- Protein-ligand interactions facilitate understanding drug design.
- There are many compounds act as ligands that could be used as a drug.
- **Ligand** binding **interactions** changes the **protein** state and **protein** function.
- The ligand is chosen by a computational process called 'Molecular Docking'.
- As below:

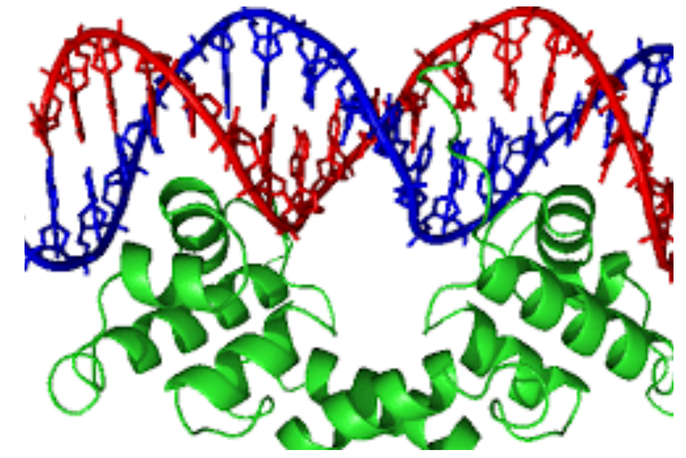


An example HIV-1 Protein-ligand interactions: protease structure in complex with the small molecule indinavir.

12

DNA or RNA-protein interactions

- **DNA-protein interactions** include those between **DNA** and transcription factors or other regulatory **proteins**.
- **RNA-protein interactions** include those between **RNA** and the ribosome, and other **RNA-binding proteins.**



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What the next steps?

1. Expression and purification of gene as a protein.
2. Tissue purified proteins preparations.

- why we are going to learn these topics?

- In order to do functional and structural assays at molecular level.
- How can be biophysics be involved in these topics?
- All the interactions of the biomolecules are based on the biophysics science.
- All the instatements are based on the biophysics science.

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Lecture 2

Protein Expression and lysis

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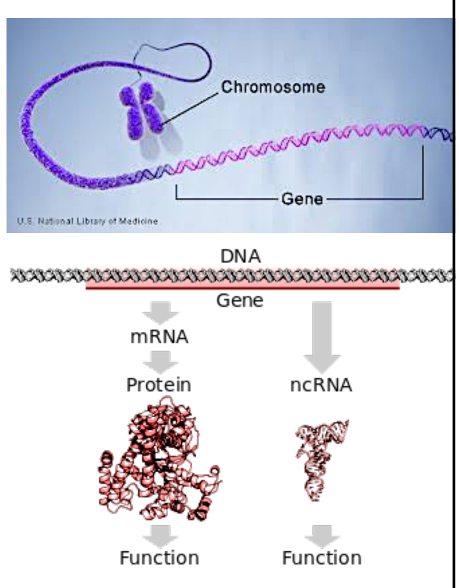
1

What is a protein expression?

- Recombinant **protein expression** refers to the manufacture of proteins derived from recombinant DNA.
- After proteins being expressed, they start to fold to make the 3D structure.
- Remember!
- **Genes** are made up of DNA.
- In biology, a **gene** is a sequence of nucleotides in DNA or RNA that encodes the synthesis of a gene product, either RNA or protein.
- Gene: A **gene** is the basic physical and functional unit of heredity.



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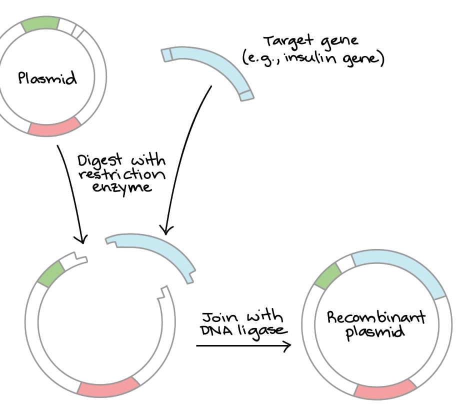
What is a gene expression?

- **Gene expression** is the process by which the information encoded in a **gene** is used to direct the assembly of a **protein** molecule.
- The cell reads the sequence of the **gene** in groups of three bases (as explained in the last lecture).
- In prokaryotic and prokaryotic, gene expression is regulated differently.
- **In prokaryotic**, Gene expression is regulated primarily at the transcriptional level.
- **In eukaryotes,** Gene expression is regulated at many levels (epigenetic, transcriptional, nuclear shuttling, post-transcriptional, translational, and post-translational).

3

To express proteins, we need a Biotechnology

- What is a DNA cloning:
- It is making an identical copy for an organism.
- It refers to the process of isolating a DNA sequence of interest for the purpose of making multiple.
- In labs, vectors are is used a a host to make an identical copy for a specific gene.
- Then, this gene is hosted in *Ecoli* produce protein (outside of the living body).
- In vivo or in vitro?



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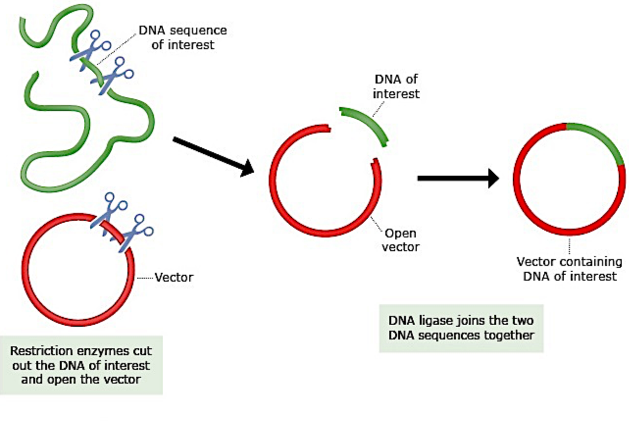
How does genetic engineering work?

- A plasmid ‘’ a sequence of DNA” is inserted to a vector which contain sites for polymerase binding that express a protein.
- Also, vector has an antibiotic sites to kill unwanted Bactria.
- It also has sites that can used to ligate or in-ligate (adhesive) the plasmid inside.
- It has also a site to induce the protein synthesis.
- Finally, vector is transformed to the Bactria ‘’a host ‘’ to synthesis the requested protein.

5

What is a genetic engineering?

- it is a removal of genes from one organism and insertion into another.



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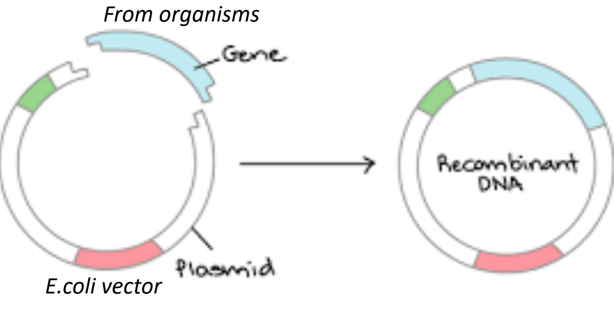
What is a recombinant DNA?

- It is DNA has been mixed with one of another species.



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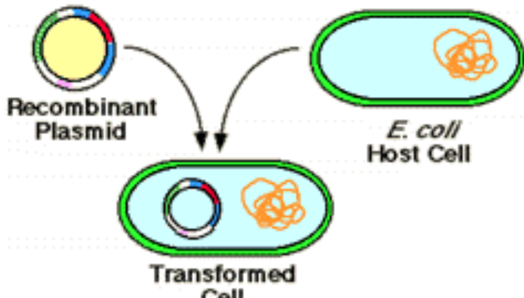
What is the transformation of a gene?

- It is a process used to transform a gene into a Bactria in order to express a protein from the selected gene.
- It is done by a adding the DNA to the Bactria and then heat shocking the Bactria at 42 ˚C in a water bath for up to 1 min only.
- Heat shocking process allow the Bactria wall to pass DNA inside it.



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The genetic engineering is done by PCR

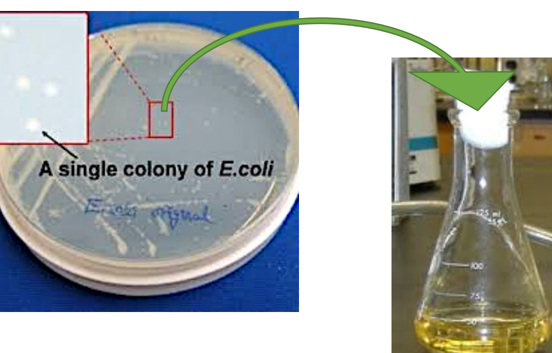
- **What is PCR technique?**
- Using Polymerase chain reaction PCR in '' three known steps'' to generate ‘' to amplify ‘' thousands to millions of copies of a DNA from a very small amount of DNA.
- **What do you need for PCR?**

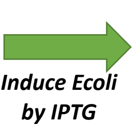
1. DNA template (the gene of interest).
2. Two primers 5' and 3'(oligo-nucleotides).
3. dNTP: Deoxy ribonucleotide triphosphate (each made up of deoxyribose sugar, phosphate group and N base).
4. Polymerase enzyme.

9

Making a growth, How?

- Once colonies of E.coli or any Bactria are grown on the plates, they can be placed into a growth media to generate millions of colonies and then induced them by IPTG to produce enough amounts of a protein.





Induce Ecoli by IPTG

An expressed protein In Ecoli

After 2h at 37˚C

10

What is the difference between an expressed protein and a tissue purified protein?

- **An expressed protein**: is a protein comes from a DNA recombinant gene transformed to a host such as Bactria or virus (Ecoli e.g). The host expresses the protein, then we can extract it and purify it in labs for experiments purposes.

- **A tissue purified protein:** is a protein comes from a specific natural tissue (muscle e.g) then it is only extracted and purified in the labs for experiments purposes.

11

How to purify a protein?

1. Tissue purified proteins.

- **We get a tissue directly from animals:**
- **E.g. Form muscle, cardiac ''heart" Skin, Nails, hairs and other parts.**

2. Expressed in E.coli.

12

How do we extract the protein form E.coli?

1- Enzymatic lysis using lysozyme.

- We can use different types of biophysics and chemical methods:
- **Enzymatic lysis** is based on the digestion of the peptidoglycan layer of the bacterial cell wall by lysozyme.

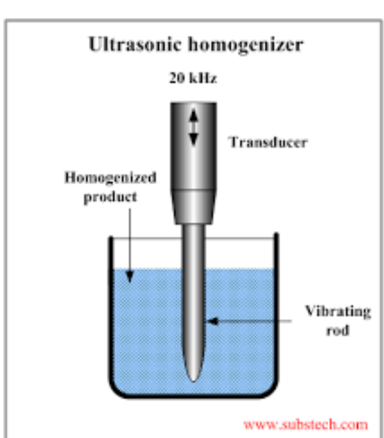
- During cell lysis often a lot of DNA is released, it becomes necessary to add DNase (1 mg/ml) to reduce the viscosity of the preparation.

13

2-Sonication

Sonication : Ultrasound waves to disrupt the cell walls.

- Cells are lysed by liquid cavitation.
- What are the Problems of this method?
- Waves increase the temperature of solution which leads to protein degradation.
- So, the solution needs to be in ice always.



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3- Freezing and grinding.

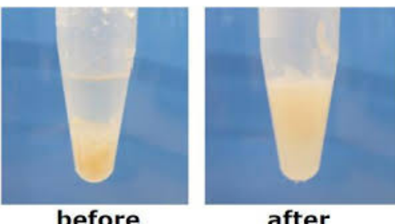
An alternative lysis method is to freeze the cells directly in liquid nitrogen and ground the frozen cells to a powder.

• Liquid N2: -80 Celsius.

15

4- Homogenization:

- **Homogenization:** The presses lyse cells by pressurizing the cell suspension and suddenly releasing the pressure.
- **French press** homogenizer is used to compact the cell by 6000-10,000 psi (pressure) to disrupt the cell walls.



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What are the optimal conditions of the biomolecules? Protein?

- Physiological environments (as inside of a living cell) .

1. The temperature 37 °C.
2. The buffer range 7-7.2.

- So, a change on these factor will lead to:

1. Protein degradation. (mis folding)
2. Protein denaturation. (mis folding)
3. Protein aggregation. (mis folding)

- Other factor could affect the protein function and structure?.
- Increase:

1. The temperature 37 °C.
2. The buffer range 7-7.4.
3. Also, UV light radiation.
4. Digestion enzymes: e.g Trypsin.

Lecture 3

Protein purification

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1

Protein purification

- Why do we purify proteins?
- To examine the function of the structure of the protein perfectly.
- If you have a mixture of proteins in a solution, then we need to purify it to have only one protein that function independently. Why?
- Other proteins and un-wanted molecules might have an impact on the protein function and structure understudy.

2

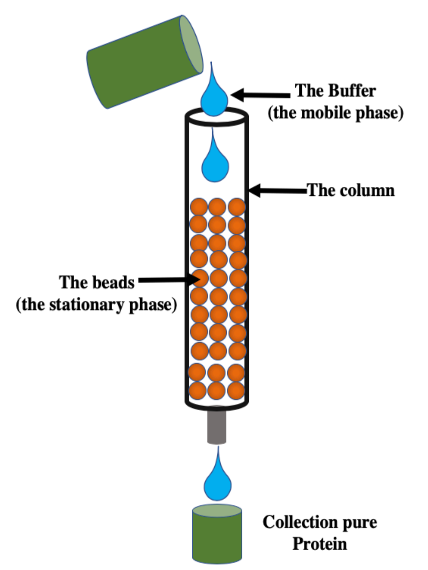
How do we purify proteins?

- Once the Cells are lysed (from the previous lecture), the mixture is then loaded to a specific Column to analyze or purify a mixture of proteins.
- What is the technique that is used to purify proteins?
- Chromatography.
- FPLC system is now used to purify proteins.
- What is a FPLC ? Previously HPLC?
- Fast performance liquid chromatography. (High)

3

Tools and Techniques: Purification & analysis of Protein

- Since we got the (extracted from the Bactria)
- **Chromatography** is used to purify protein.
- It uses a **column** packed with a porous matrix (**the stationary phase**) and a buffered solution (**the mobile phase**)
- ***Proteins*** *or other solutes pass through the column at different rates, de- pending on how they interact with the stationary phase.*



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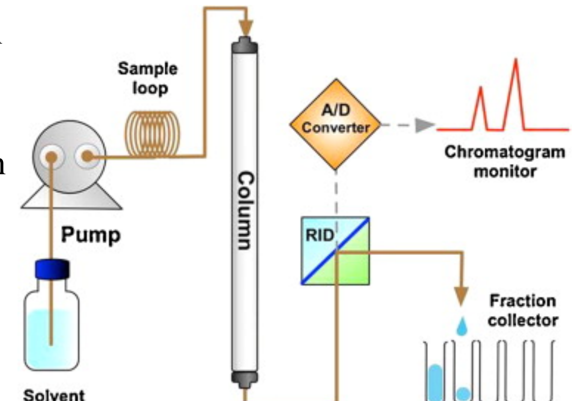
What is the Fast performance liquid chromatography.

Column

- The method is based on loading or injecting the mixture of the protein into a specific column (where the protein can bind to its resin)
- Then it can be eluted by another solution to collect it and analyze it.
- Pump (with controlled pressure) is used to for loading.



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All purification methods are based on the same idea!

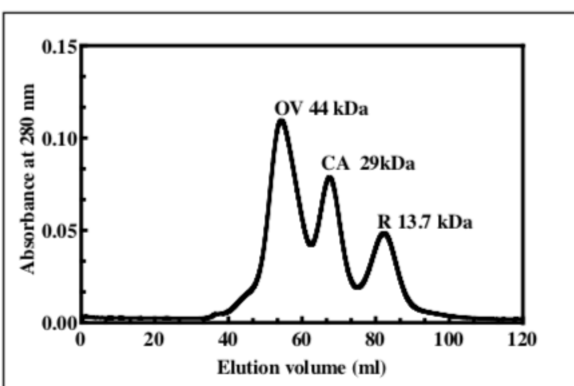
Equilibrate Load sample Wash Elute

6

FPLC/HPLC

- Modern systems use an internal detector to show the chromatogram which shows the peaks of each protein.
- The purified protein can be fractionated and collected in small tubes for further analysis.

Give a chromatogram example of Separation three proteins by FPLC?



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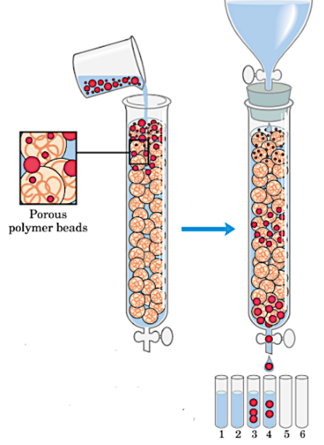
How can we choose the column to purify protein?

- We choose the columns depends on the ability of protein to bind the resins of the column.
- The resins of the columns have specific dimeter.
- Each column has different properties.
- So, lets know….



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The types of columns?

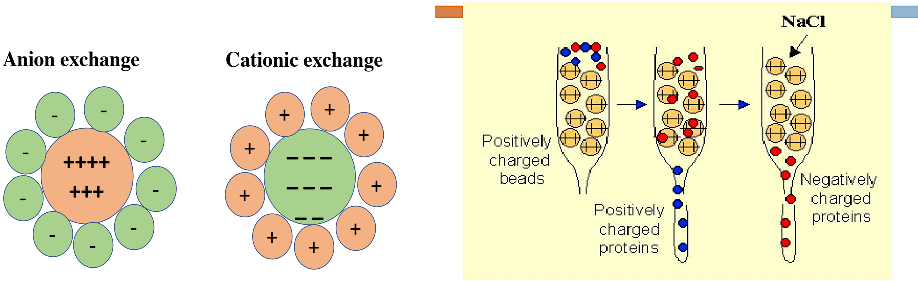
- What are the different common types of columns?

1. Ion exchange IXC chromatography .
2. Gel filtration GF chromatography.
3. Hydrophobic interaction chromatography HIC.
4. Affinity chromatography.

9

Ion exchange IXC chromatography .

- It separates the compounds according to the nature and degree of their ionic charge.
- Anion exchange resins have positive charge and used to separate negatively charged molecules.
- Cation exchange resins have a negative charge and are used to separate positively charged molecules (like SP column).



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Gel filtration GF chromatography

- In this type, the protein is separated in base of molecule size to which the molecules have different degrees of access.
- The smaller molecules have greater access, but the large molecules are excluded from the matrix (elute first).
- It is also called ‘’ size exclusion chromatography’’

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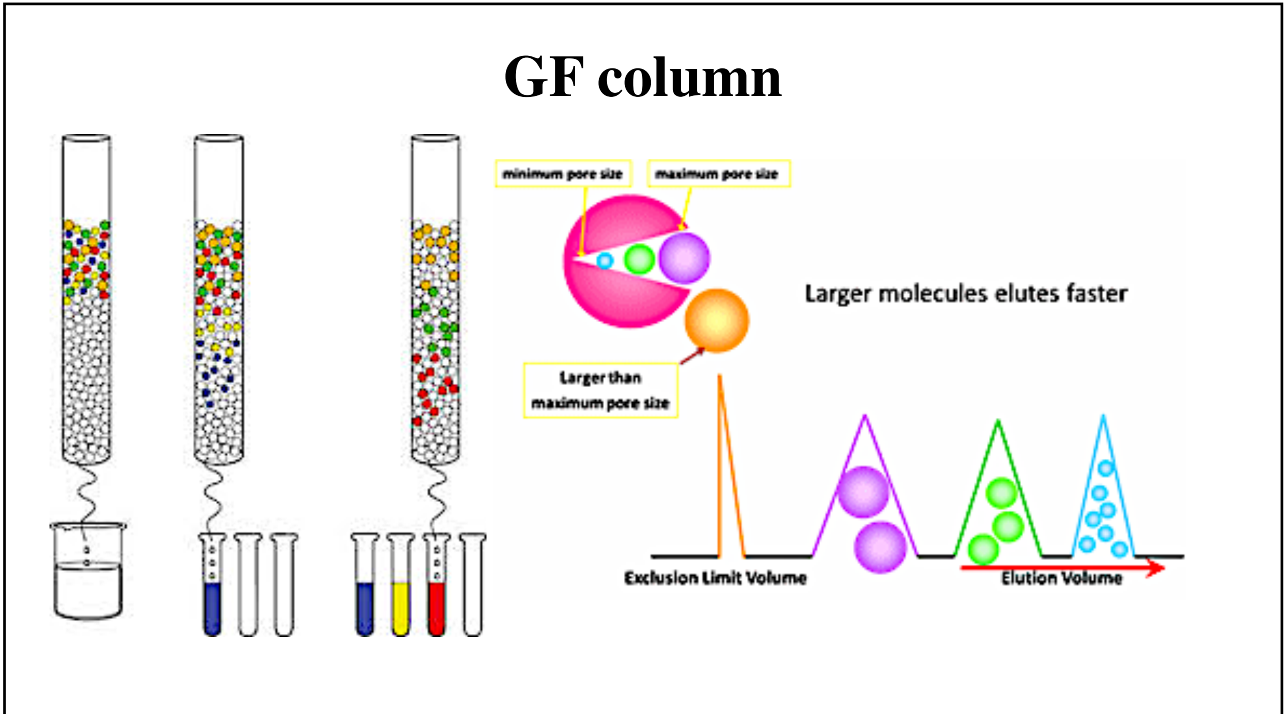
GF column



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Hydrophobic interaction chromatography HIC.

- HIC media is amphiphilic (means a protein has both hydrophilic and hydrophilic) to allow the separation on proteins based on their surface hydrophobicity.
- In the low salt buffer, interaction between the hydrophobic region and the resin is very weak, however, the interaction is enhanced in a high ionic strength buffer.

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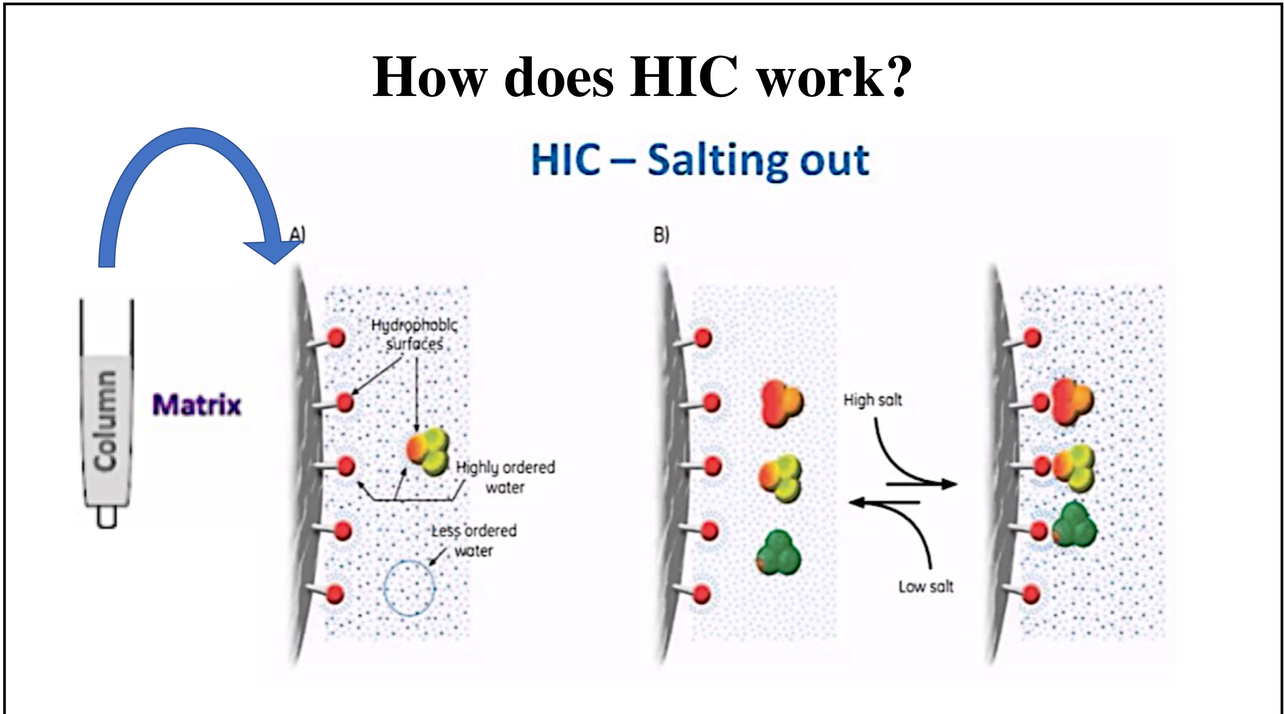
How does HIC work?



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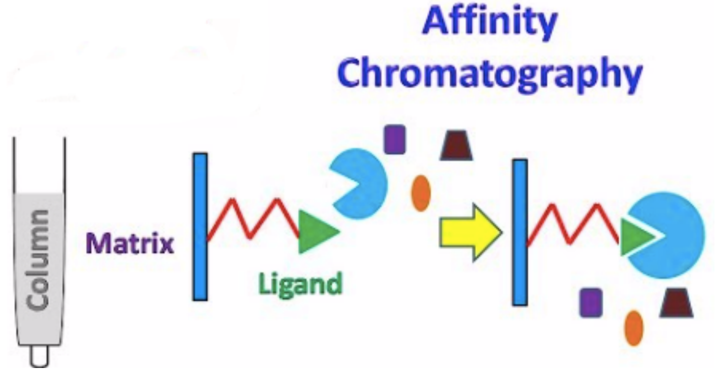
Affinity chromatography:

- The resins have ligands attached to their surfaces which are specific for a particular molecule.



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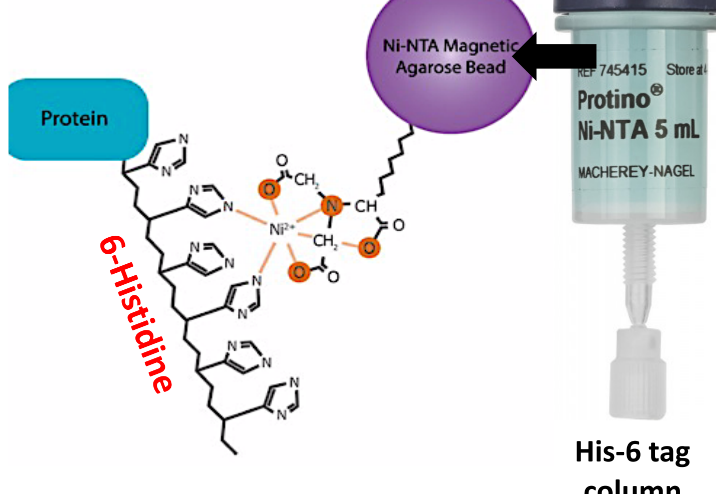
His 6-tag method

- What is his-tag method?
- It is a type of affinity chromatography.
- What is based on?
- Ni Nickle on the resin binds to specific molecules (six 6 Histidine residues that are attached to the protein).
- Histidine residues can coordinate to make a complex with Nickle (transition metal).

16

Describe the chemical binding of the His-6- tag

1. The Ni makes six bonds with the ligands like indole ring.
2. So, the Histidine contains an indole ring.
3. The 6 histidine binds the Ni on the column.
4. Un-wanted protein are washed by a buffer.
5. The pure protein is eluted and collected.



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Describe the tag process?

- Before the purification, the 6 H histidine (amino acid molecules) need to be genetically engineered and connect them to the protein.
- After the purification, they need to be cut out ‘’ cleaved out’’ by an enzyme called TEV.

18

Last but no the least

1. The purity of the protein must be checked. How?
2. The molecular weight MW of the protein must be analyzed and conformed at the expected size. How?
3. The buffer solution needs to be changed sometimes. How?
4. The protein concentration needs to be measured. How?
5. The low concentration protein needs to be concentrated. How?
6. Finally, the protein needs to be frozen and stored at -20 to -80 ˚C. How?

19

Lecture 4

Examination of protein purity

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1

What is next after purifying protein?

1. The purity of the protein must be checked. How?
2. The molecular weight MW of the protein must be analyzed and conformed at the expected size. How?
3. The buffer solution needs to be changed sometimes. How?
4. The protein concentration needs to be measured. How?
5. The low concentration protein needs to be concentrated. How?
6. Finally, the protein needs to be frozen and stored at -20 to -80 ˚C. How?

2

How to analyze the purity of the protein?

- Using Gel-electrophoresis SDS-PAGE method.
- What is the SDS method for?
- SDS-PAGE is an analytical technique to separate proteins based on their molecular weight
- What is based on?
- The protein migrates by applying an electric field allowing the protein to pass through the pores inside the matrix (gels).
- Why it is called SDS PAGE?
- Because the chemical ”SDS” is used to denture the protein and allow it to pass through the gels holes.

3

SDS page and equipment

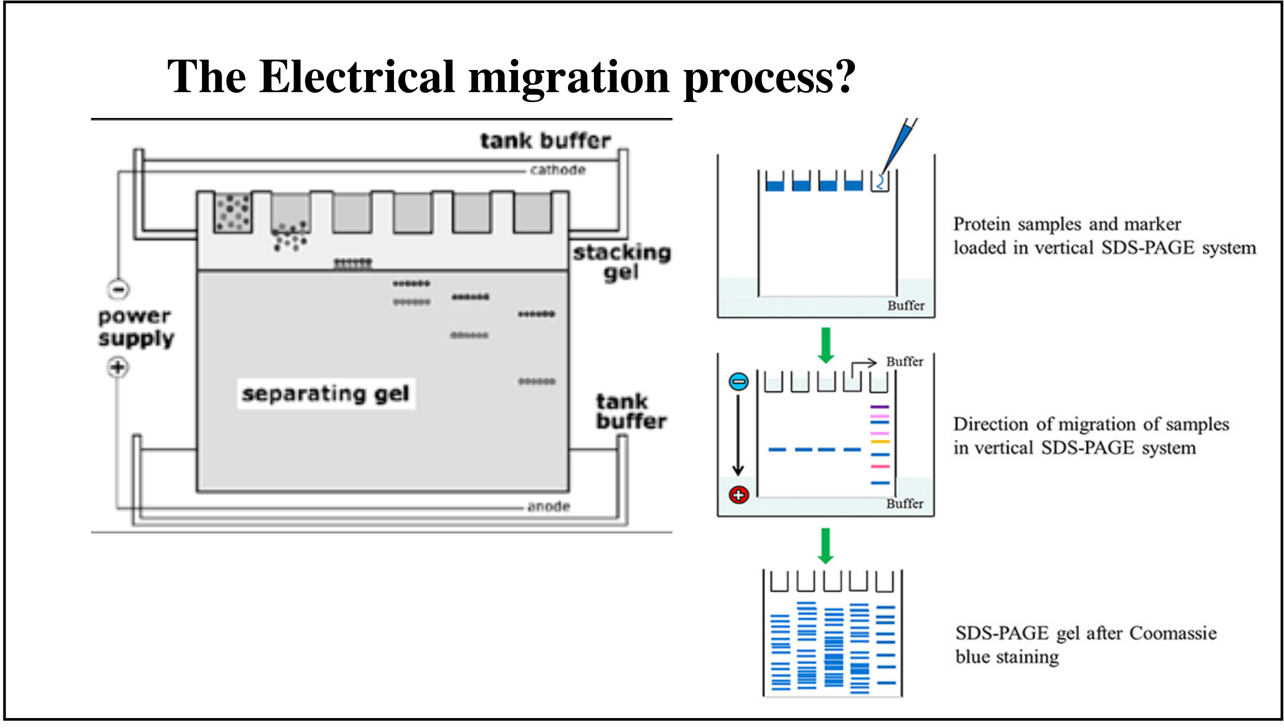
4

Describe the SDS-page?

1. The full SDS page contains two gels layer.
2. The large gel called separating gel.
3. The small gel called staking gel.
4. The full page then immerged in a glycine buffer.
5. An electrical field is applied for migration.
6. The large protein will stop first, the small protein will take longer.
7. A protein marker is used to analyze the gel.
8. The gel is the then stained by a dye to show the band of the protein.

5

The Electrical migration process?



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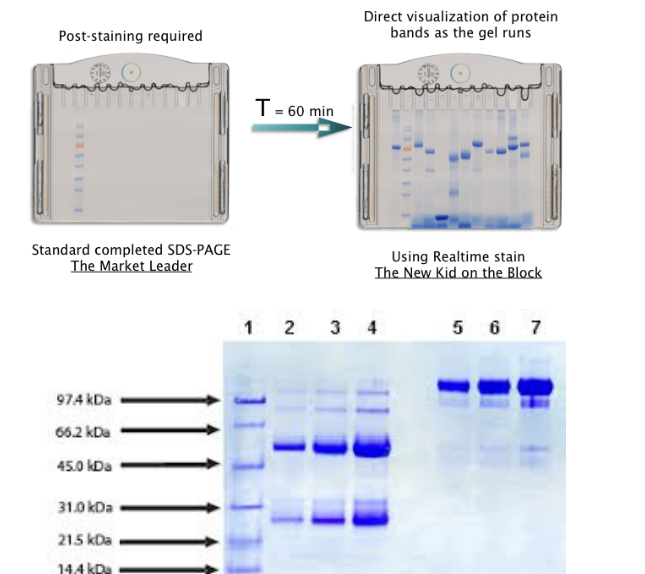
Describe this SDS page ? The MW?

1. Is the protein pure?
2. How many bands in the single lane?
3. Is it one protein in a lane or more? complex?
4. What is the purity % of the protein?
5. What is the MW of each band?
6. https://www.youtube.com/watch?v=i_6y6Z5UvwE



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What is the Dialysis?

It is a physical phenomena allows diffusion between two liquid media.

Dialysis step allows for:

1. Buffer exchange.
2. removal of salts or other contaminants to the protein samples.
3. It is typically carried out using various forms of semi-permeable membranes and tubing.
4. Due to the pore size of the membrane, large molecules in the sample cannot pass through the membrane.
5. Each tube has special MWCO (molecular weight cut off) !! Which must be less than half of the MW of the protein in order to avoid loosing protein.

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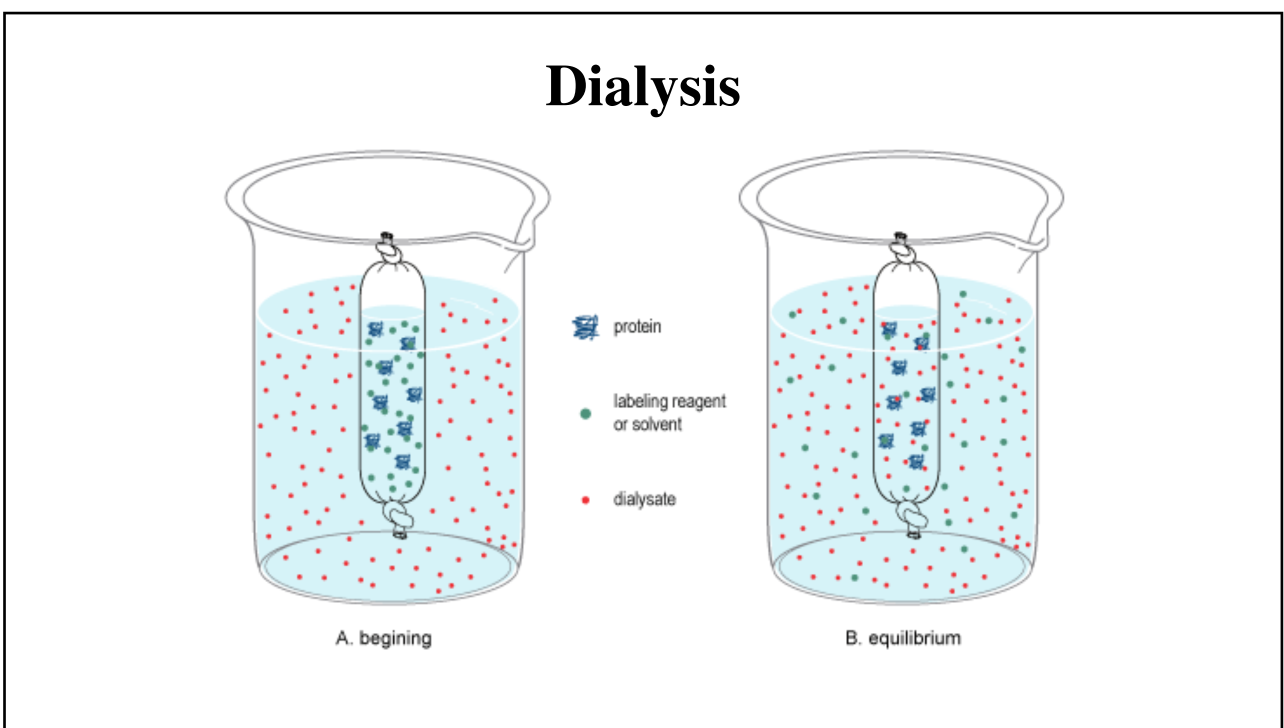

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How do you measure the protein concentration?

- There are several methods to measure the Conc.
- Measure the Absorption A or called Optical density O.D using spectrophotometer at 280 nm wavelength.
- In this method, we need to know:
- The molar absorptivity E of the protein by calculating the number of aromatic amino acids in the protein which are Tryptophan and Tyrosine (in the websites). Why?
- Because the aromatic ring gives absorption at 280 nm.

10

So, what ?

- So, what does that mean?
- Means: from Beer-lambert equation which is (A=E x b x c) we can calculate the concentration.
- A= absorption.
- E= molar absorptivity.
- b= cuvette width usually 1 cm.
- C=the concentration

- For example:
- If the molar absorptivity protein = 0.25 g/L
- And we read the O.D (optical density) e.g. A=1
- (A=E x b x c) 1= 0.25 x1 xc
- c=4 mg/ml

11

What about if the protein is diluted?

- The protein buffer needs to concentrate.
- There are several ways to concentrate protein:

1. lyophilization method:
2. precipitation by Ammonium sulphate.
3. Using filter tubes and others.

12

Lyophilization method:

- Describe the lyophilization method?
- It is a method used for concentrating and powdering proteins.
- What should I do to protein before lyophilization.
- The protein must be flash frozen by liquid N2
- How does it work?
- It works by a sublimation in the freeze-drying process.

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Precipitation by Ammonium sulphate.

- Describe concentrating protein by NH_2SO_4 ?
- it stabilizes proteins.
- It precipitates the proteins by salting out at high ionic strength. Thus, the protein will be concentrated.
- Protein could be re-solubilized in a buffer and then dialyzed multiple times. Why? To get red of the salts.

14

How do you store the protein?

- Protein must be frozen by Liquid N2 ?
- Why?
- Because it flashes freeze the protein very quickly which avoids the degradation in the structure.
- Usually, protein stored for a short time at -20 ˚C.
- But for long time, it must be stored at -80 ˚C.

15

Lecture 5

Protein Crystallization and x-ray structure

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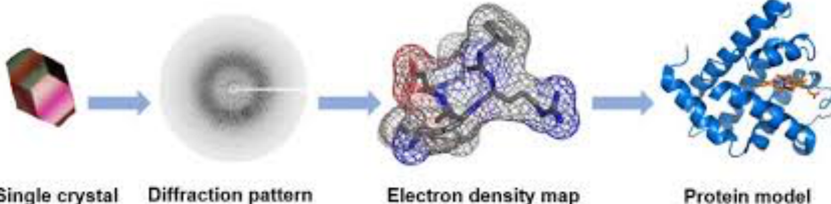
1

Protein Crystallization

- There many methods can observe the protein structure ?
- So, Why do we need to crystalize a protein?
- To observe the shape ‘’structure ” of the protein in the crystal.
- So, what is the phase of the crystal?
- Rigid solid.



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How to get a good crystal structure?

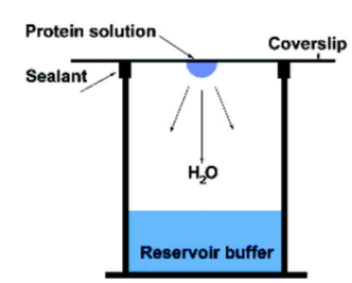
- When to get a best structure produced by X-ray of a protein?
- the best structure produced by X-ray when the molecules are well crystalized.
- What does protein crystallization require?
- a formation of large and stable crystals.
- How do we crystalize e protein molecule?
- there are two experimental methods used to form crystals from protein solutions are

1. vapour diffusion
2. equilibrium dialysis.

3

Vapour diffusion

- Vapour diffusion : it is based on ‘hanging drops’ containing protein solution plus ‘precipitant’ at a concentration insufficient to precipitate the protein.
- The drop is equilibrated against a larger reservoir of solution containing precipitant.
- after sealing the chamber equilibration leads to supersaturating concentrations that induce protein crystallization in the drop.

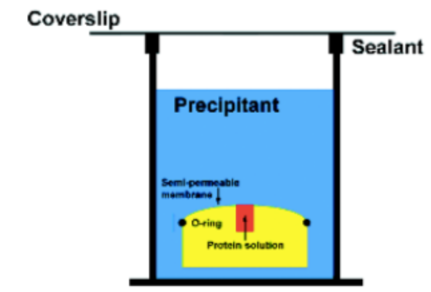


The ‘hanging drop’ or vapour diffusion method of protein crystallization. As little as 5 μl of concentrated solution (protein + solvent) may be suspended on the coverslip

4

equilibrium dialysis

- The equilibrium dialysis method is used for crystallization of proteins at low and high ionic strengths.
- Small volumes of protein solution are placed in a container separated from precipitant by a semi-permeable membrane.
- Slowly the precipitant causes crystal formation within the well containing the protein solution.



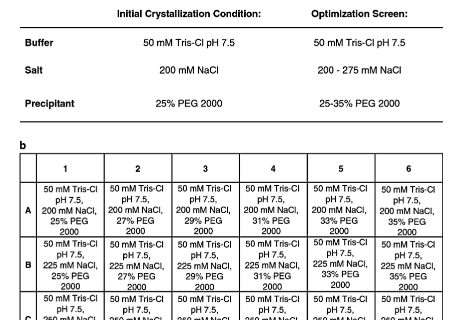
Equilibrium dialysis can be achieved with many different 'designs' although the basic principle involves the separation of protein solution from the precipitant by a semipermeable membrane. Diffusion across the membrane promotes ordered crystallization

5

Optimization the conditions for the crystallization

- **Optimization means an** improvement process.
- It involves sequential and incremental changes in the chemical and physical parameters that influence **crystallization**.

1. The The chemical parameters: pH, ionic strength and precipitant concentration.
2. The physical parameters such as temperature, sample volume and overall methodology.



a

	Initial Crystallization Condition:	Optimization Screen:
Buffer	50 mM Tris-Cl pH 7.5	50 mM Tris-Cl pH 7.5
Salt	200 mM NaCl	200 - 275 mM NaCl
Precipitant	25% PEG 2000	25-35% PEG 2000

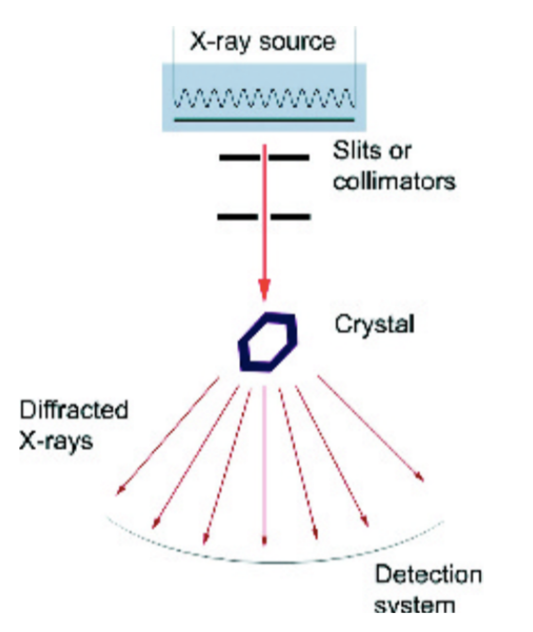
b

	1	2	3	4	5	6
A	50 mM Tris-Cl pH 7.5, 200 mM NaCl, 25% PEG 2000	50 mM Tris-Cl pH 7.5, 200 mM NaCl, 27% PEG 2000	50 mM Tris-Cl pH 7.5, 200 mM NaCl, 29% PEG 2000	50 mM Tris-Cl pH 7.5, 200 mM NaCl, 31% PEG 2000	50 mM Tris-Cl pH 7.5, 200 mM NaCl, 33% PEG 2000	50 mM Tris-Cl pH 7.5, 200 mM NaCl, 35% PEG 2000
B	50 mM Tris-Cl pH 7.5, 225 mM NaCl, 25% PEG 2000	50 mM Tris-Cl pH 7.5, 225 mM NaCl, 27% PEG 2000	50 mM Tris-Cl pH 7.5, 225 mM NaCl, 29% PEG 2000	50 mM Tris-Cl pH 7.5, 225 mM NaCl, 31% PEG 2000	50 mM Tris-Cl pH 7.5, 225 mM NaCl, 33% PEG 2000	50 mM Tris-Cl pH 7.5, 225 mM NaCl, 35% PEG 2000
C	50 mM Tris-Cl pH 7.5, 250 mM NaCl, 25% PEG 2000	50 mM Tris-Cl pH 7.5, 250 mM NaCl, 27% PEG 2000	50 mM Tris-Cl pH 7.5, 250 mM NaCl, 29% PEG 2000	50 mM Tris-Cl pH 7.5, 250 mM NaCl, 31% PEG 2000	50 mM Tris-Cl pH 7.5, 250 mM NaCl, 33% PEG 2000	50 mM Tris-Cl pH 7.5, 250 mM NaCl, 35% PEG 2000
D	50 mM Tris-Cl pH 7.5, 275 mM NaCl, 25% PEG 2000	50 mM Tris-Cl pH 7.5, 275 mM NaCl, 27% PEG 2000	50 mM Tris-Cl pH 7.5, 275 mM NaCl, 29% PEG 2000	50 mM Tris-Cl pH 7.5, 275 mM NaCl, 31% PEG 2000	50 mM Tris-Cl pH 7.5, 275 mM NaCl, 33% PEG 2000	50 mM Tris-Cl pH 7.5, 275 mM NaCl, 35% PEG 2000

6

X-ray method

- Once we get the crystal, How can we see the shape ''structure '' of the protein in a crystal?
- By applying an X- ray source incident on a crystal located close to a detector. This leads to reflect the X-rays by series of atomic planes.



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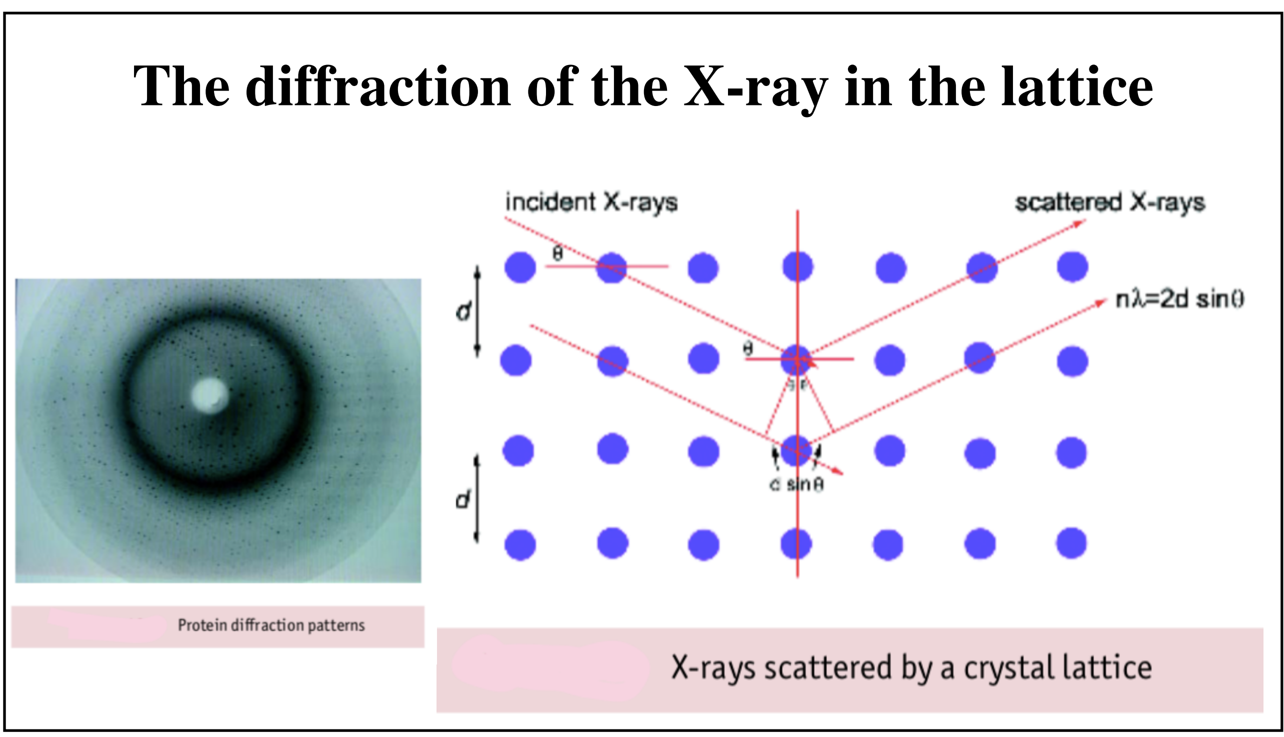
Bragg's law

- Who has developed the x-ray method?
- Bragg.
- What does it explain ?
- It explains the the angles measurements when an incident X-ray beam is diffraction by a atoms of the molecules which leads to scatter the beam by the crystal lattice.
- What is the Bragg's equation?
- $n\lambda = 2d \sin \theta$

8

The diffraction of the X-ray in the lattice



Protein diffraction patterns

X-rays scattered by a crystal lattice

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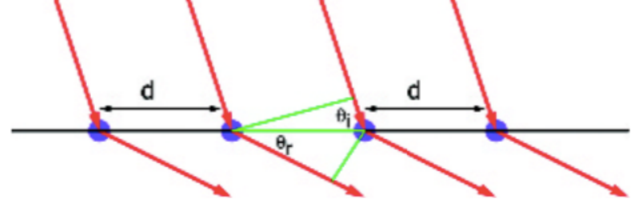
Laue equation

- Laue arranged the eq: $d\cos\theta_i - d\cos\theta_r = n\lambda$ (where n=1,2,3,...) to calculate three dimensions 3D.



The Laue equations

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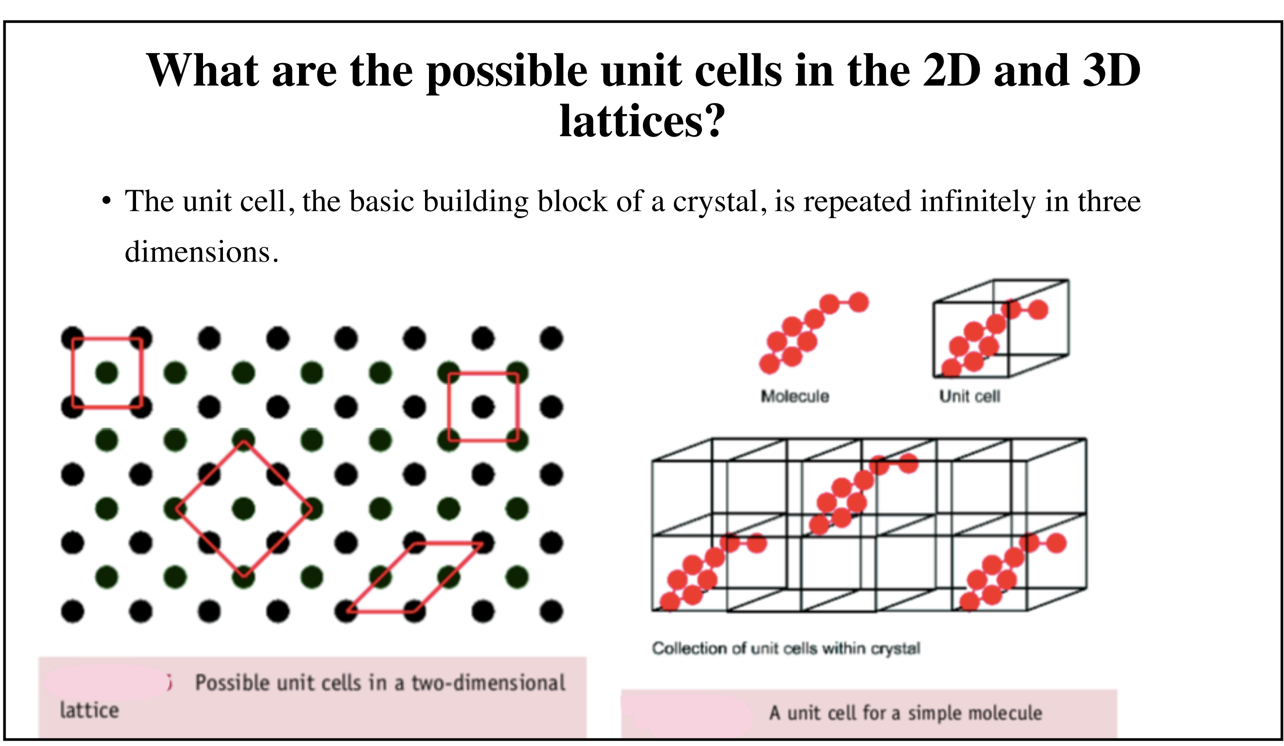

Possible unit cells in a two-dimensional lattice

A unit cell for a simple molecule

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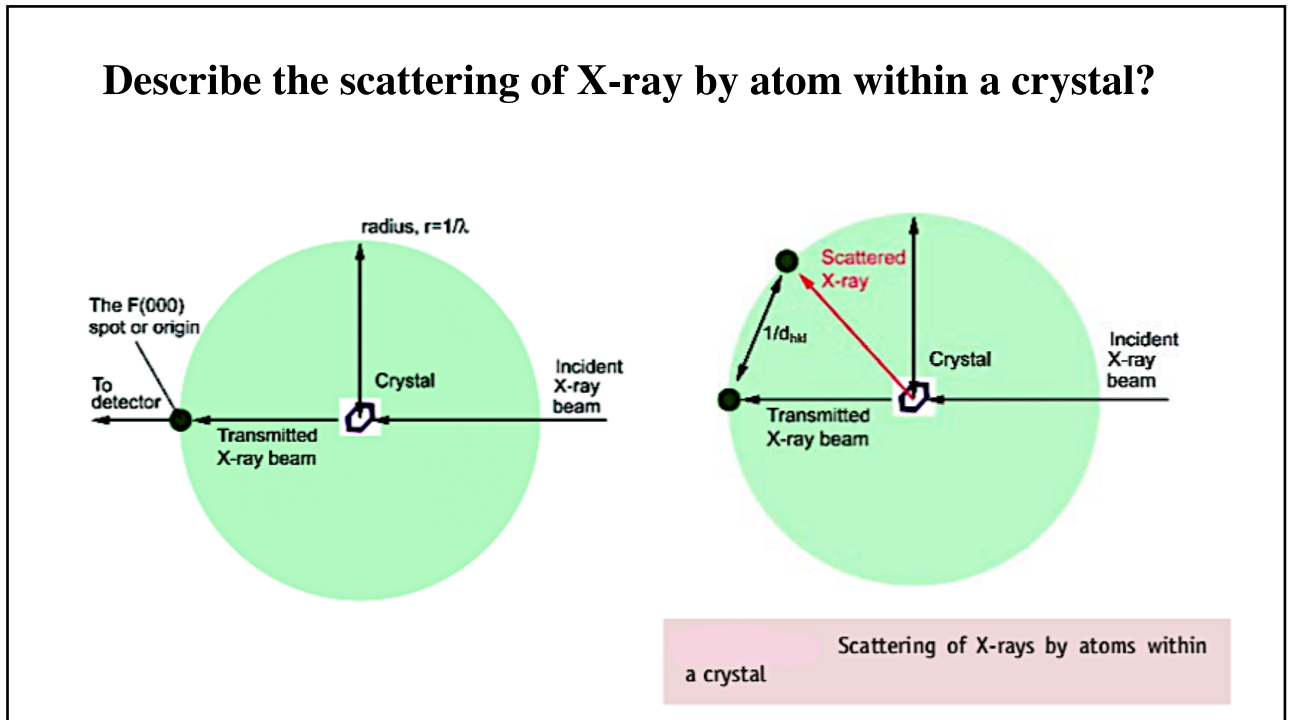

Scattering of X-rays by atoms within a crystal

12

With rotation !

-201 -101 001 101

Crystal

Incident X-ray beam

-200 -100 000

Transmitted X-ray beam

-20-1 -10-1 00-1 10-1

Rotation of the crystal brings more planes (collections of atoms) i

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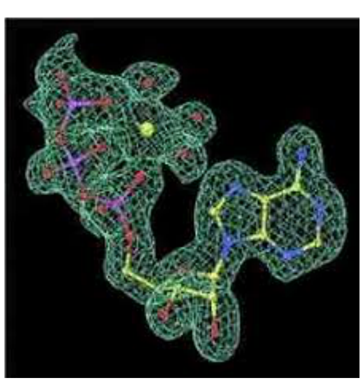
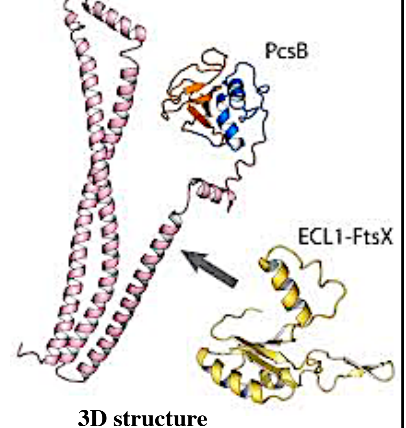
Proceeding data:
From electron density map to a protein modeling and structure.

- Once the data of the x-ray diffraction is collected, different software are used to process the data for protein molding.



Electron density map



3D structure

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What is the main purpose of solving the 3D structure?

- What is the main purpose of solving 3D structure of protein?

1. **To study protein structure features.**
2. **For drug design.**
3. **To study biomolecules interactions.**

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Biomolecule interactions

- **How can we study the protein-protein interaction via crystallization?**
- The protein in the crystal **shows** number of bonds that **can** form with another **protein** through intermolecular interactions.
- So, these interactions depend on electron densities of molecules and the **protein** side chains that change as a function of pH.

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Lecture 6

Protein NMR.

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1

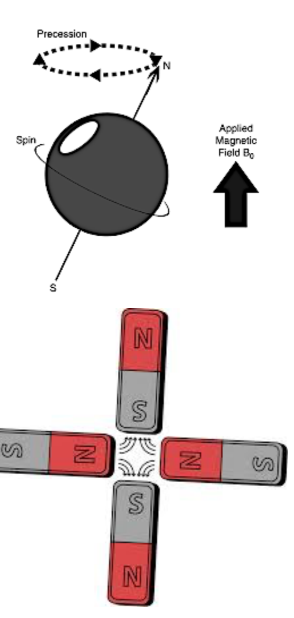
Overview

- Do you remember?
- Protein consists of hundreds amino acids.
- Amino acids are made mainly from organic compounds which consists of C, N, O and H.
- Meaning: these compounds can be characterized by NMR nuclear magnetic resonance **spectroscopy** as same as any other organic compounds.

2

NMR phenomena

- At NMR phenomenon: the property of all atomic nuclei becomes 'spin'.
- Spin describes the nature of a magnetic field surrounding a nucleus and is characterized by a spin number.



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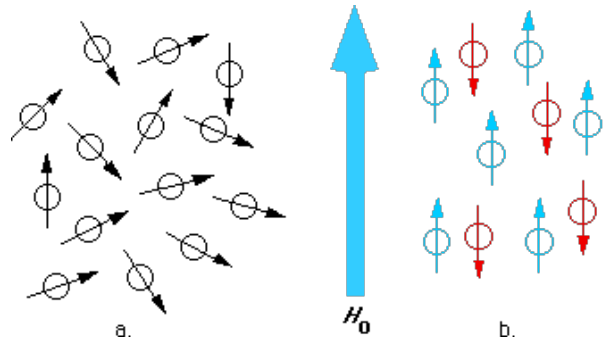
What happen if you apply a magnetic field on atoms.

- To produce transitions between the energy levels>
- When we apply a magnetic field to electrons, they will align either with magnetic filed direction or against it. Why?



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Magnetization

- How magnetic field of the element can be active or silent?
- Spin 1/2 nuclei represent the simplest situation and arise when the N+P=odd number 1,3,5…. is an odd number:
- ^{12}C the most common isotope is NMR ‘silent’ and the ‘active’ spin 1/2 nucleus (^{13}C).

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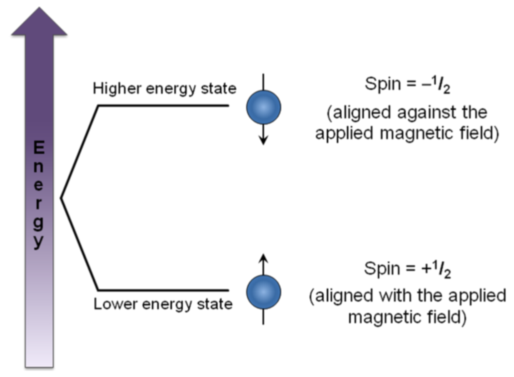
Energy of spinning

- Spins aligned parallel with external magnetic fields are of slightly lower energy than those aligned in an antiparallel orientation.
- to predict transitions between lower and higher energy level.



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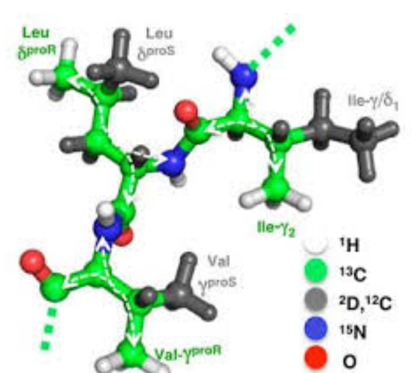
Isotopes



- 1H proton is not an isotope but it is active.
- 2D Deuterium is an isotope but it silent.
- 12C proton is not an isotope but it is silent.
- 13C proton is an isotope but it is active.
- 14N proton is not an isotope but it is silent.
- 15N proton is an isotope but it is active.

7

What we need to get an active protein in the NMR?

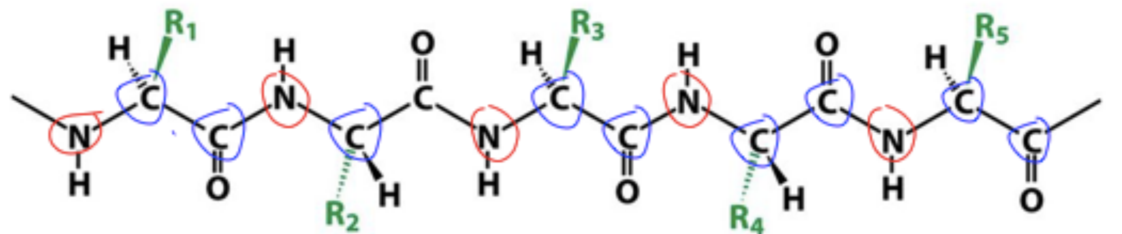
- What we need to get an active protein in the NMR?
- So, the protein must have expressed in isotopes such as ^{15}N and ^{13}C.
- HOW?
- By growing up the bacteria in an isotope media instead of LB media (normal) to produce labeled protein.
- Bactria eat the isotope N or C to express protein.

amino terminus (N terminus)
carboxyl terminus (C terminus)

Polypeptide backbone

8

What is this method called? It is called isotope labeling



The polypeptide backbone of the protein labeled with both 15N and 13C

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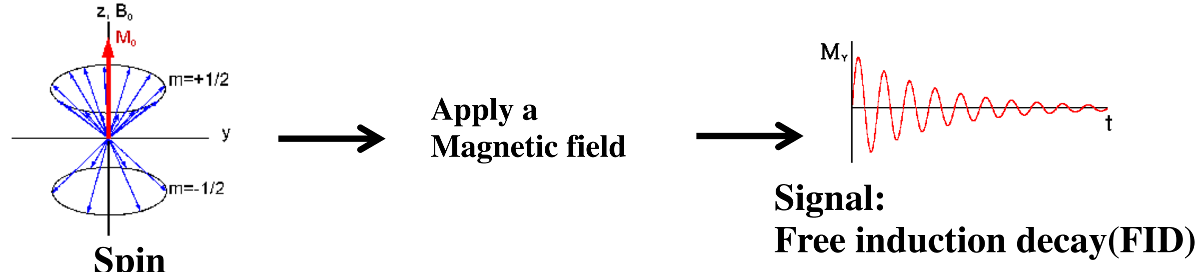
How to convert NMR signal to data



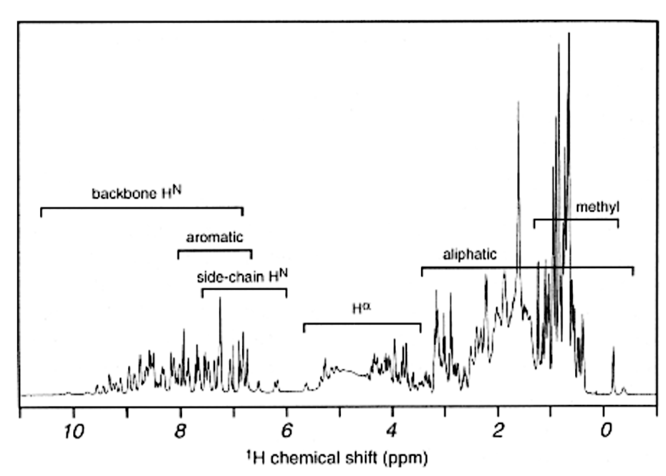
Spin → **Apply a Magnetic field** → **Signal: Free induction decay(FID)**

Analysis → **1D spectra** → **2 D spectra** → **Protein structure**

10

What is the one-dimension 1D spectra ?

- 1D spectra shows the chemical shift peaks of the atoms in different regions due to NMR fields.
- The spectra is run form right at 0 ppm to left 14 ppm..
- 0-1.7 ppm methyl groups.
- 1.7-3 ppm aliphatic compounds.
- 3-6 ppm H alpha protons.
- 6-8 ppm side chains of amino acids.
- 7-8 ppm aromatic compounds.
- 7-11 ppm N of backbone N-H and C=O.



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Example? 1D spectra of Ethanol molecule as a small

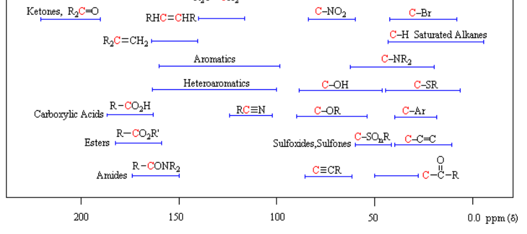
1H NMR Spectrum of ethanol

1 OH 2 CH_2 3 CH_3 TMS

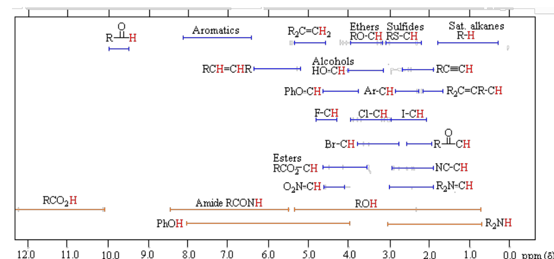
9 8 7 6 5 4 3 2 1 0

δ

Reference table for only 12C



Reference table for only proton H^+



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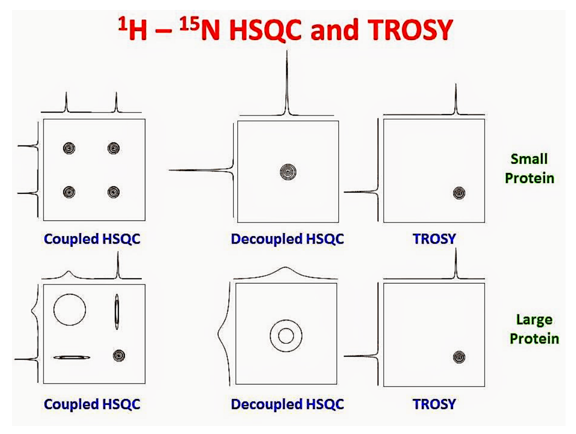
What is the 2D spectra of NMR?

- The 2D spectra eexperiments are two frequency axes representing a chemical shift. It shows the correlation of atoms with other.
- The overview spectra is as you look at the peaks from the top.



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Types of the 2D experiments

- There are many experiments of 2D. E.g:

1. COSY: it is used for small organic compounds to show the correlation between H and others in the compound.
2. HSQC and TROSY: it is used for proteins to show the correlation between H and other N of the compound.
3. NOE: it is used to show the correlation between any two H in the compound.

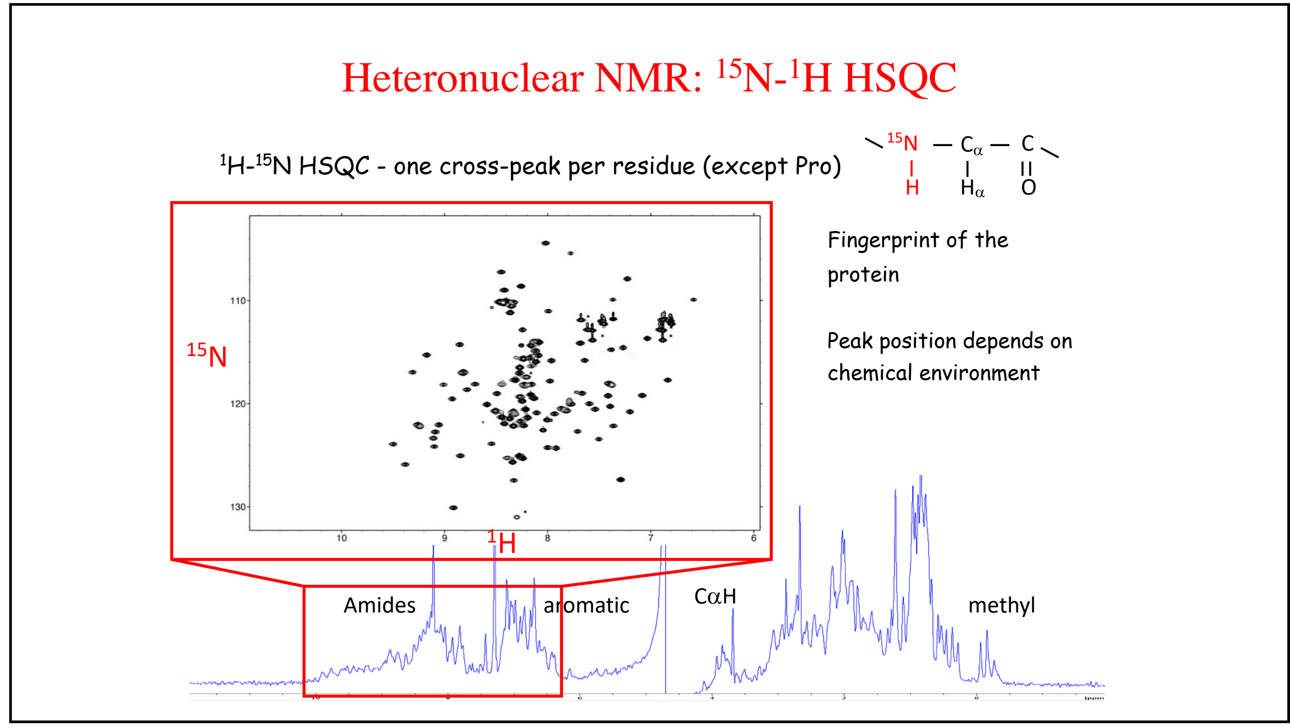
14

What do 2D HSQC spectra of protein show?

- HSQC show only the labelled atoms such as (13C and 15N) on the protein.
- Therefore, from HSQC spectra we can calculate exactly the number of amino acids in the protein.
- Also, we can assign each amino acid and and find the sequence of all.
- Sequence means: the order of the amino acids in the protein.

15

Heteronuclear NMR: ^{15}N-^{1}H HSQC



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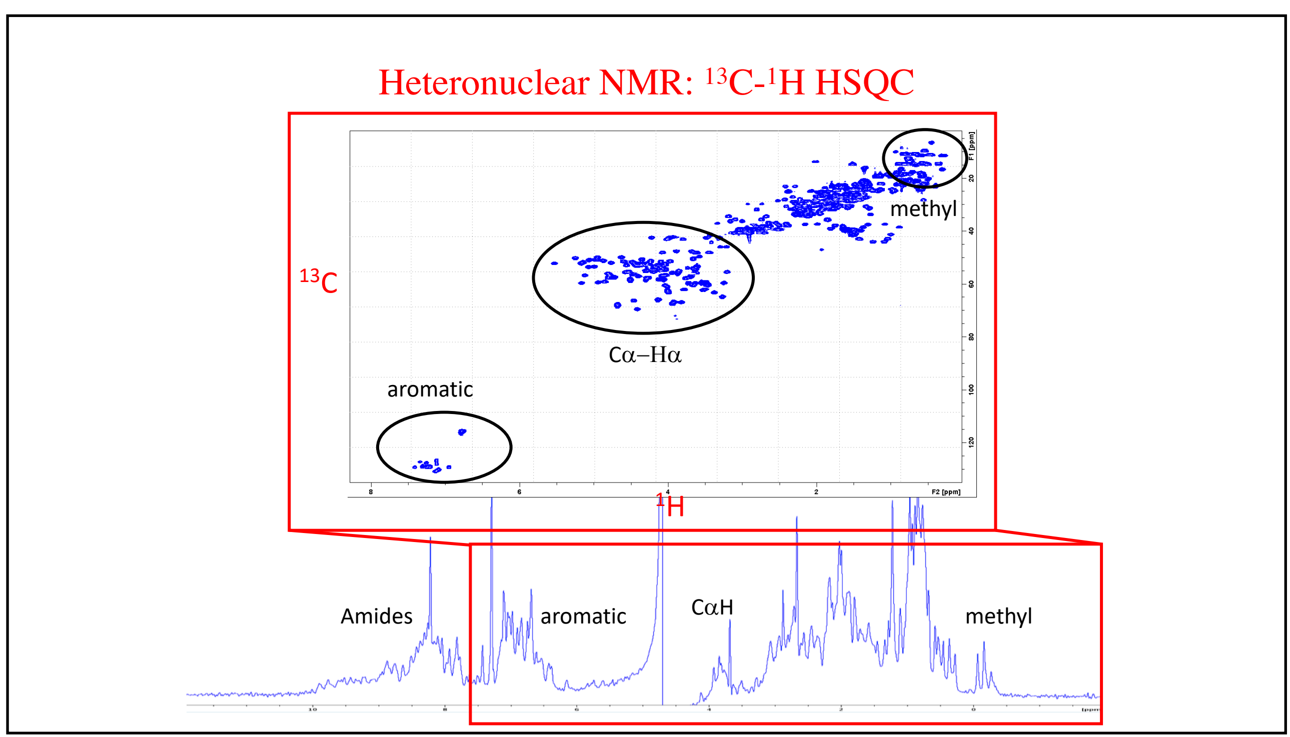

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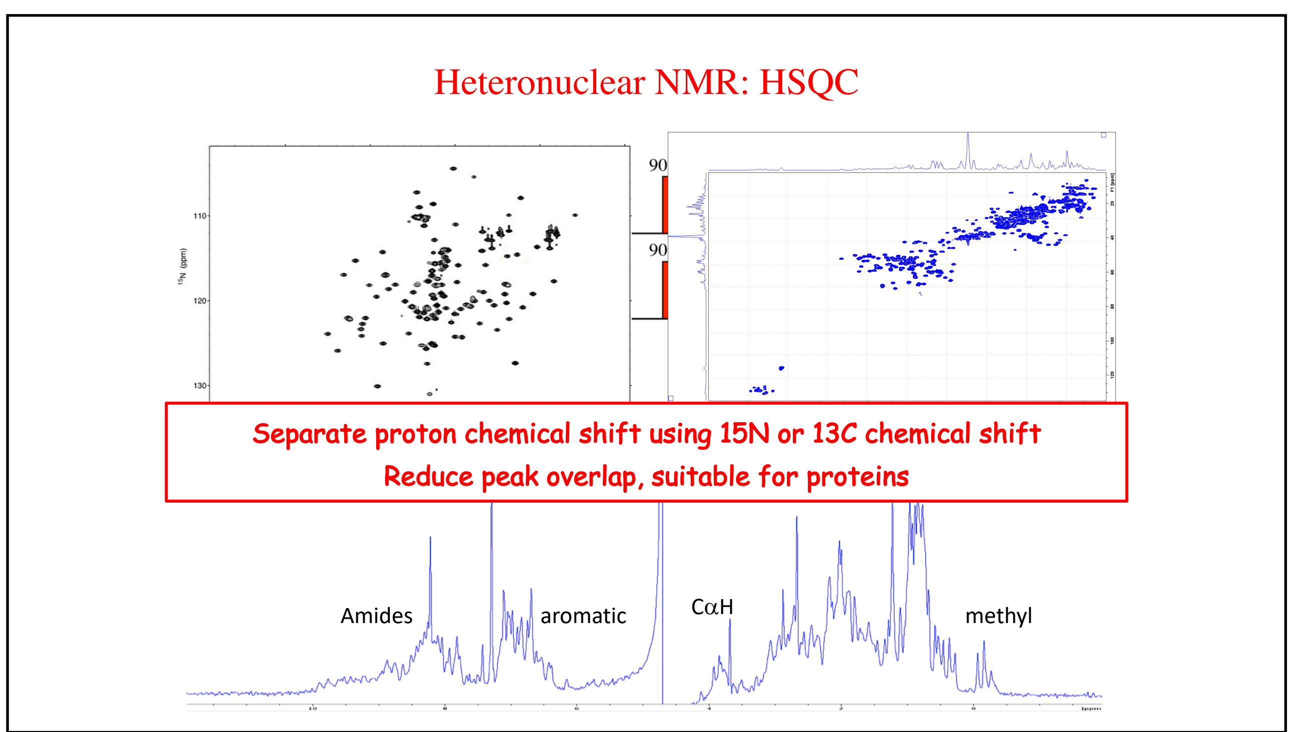

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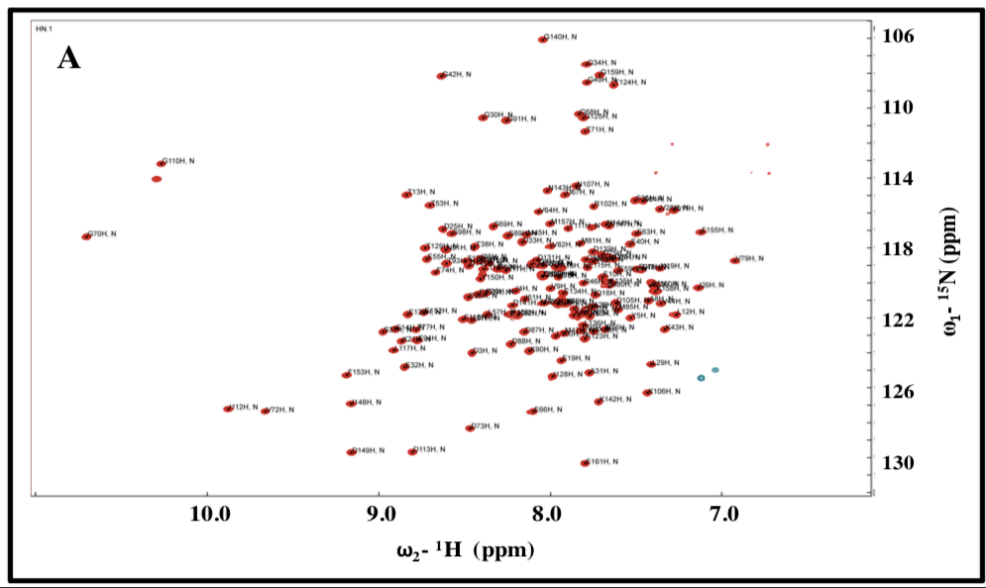
Just example: calculate the number of amino acids in the spectra?



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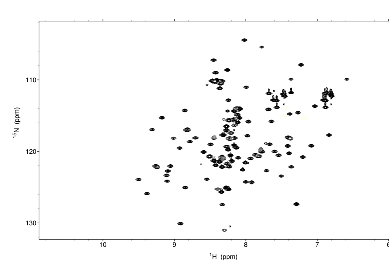
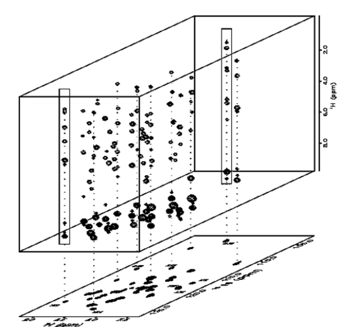
What we get from studying 3D and 4D?

- We can get the 3D structure of the protein and biomolecules.
- We can measure the length of the bonds between any two atoms in the protein.





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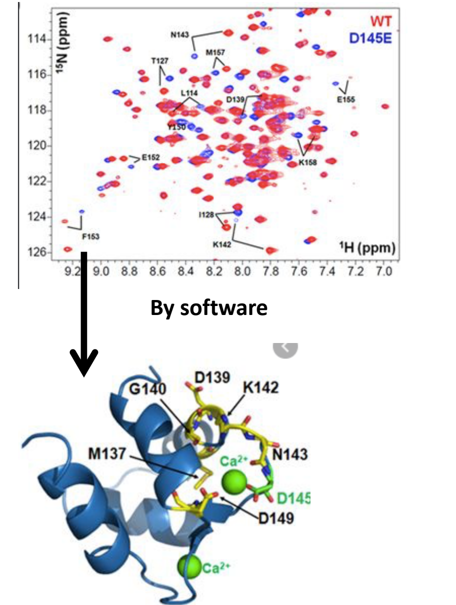
What we do get from processing NMR spectra?

- We can get the 3D structure of the protein.
- There are many pdb for protein structure are available online. Visit RCSB PDB: Homepage
- What we can study on the 3D NMR structure of the protein?

1. To study the protein-protein interactions.
2. Drug design.
3. Protein modeling.
4. Protein function.



Lecture 7

Optical spectroscopic techniques for biomolecules.

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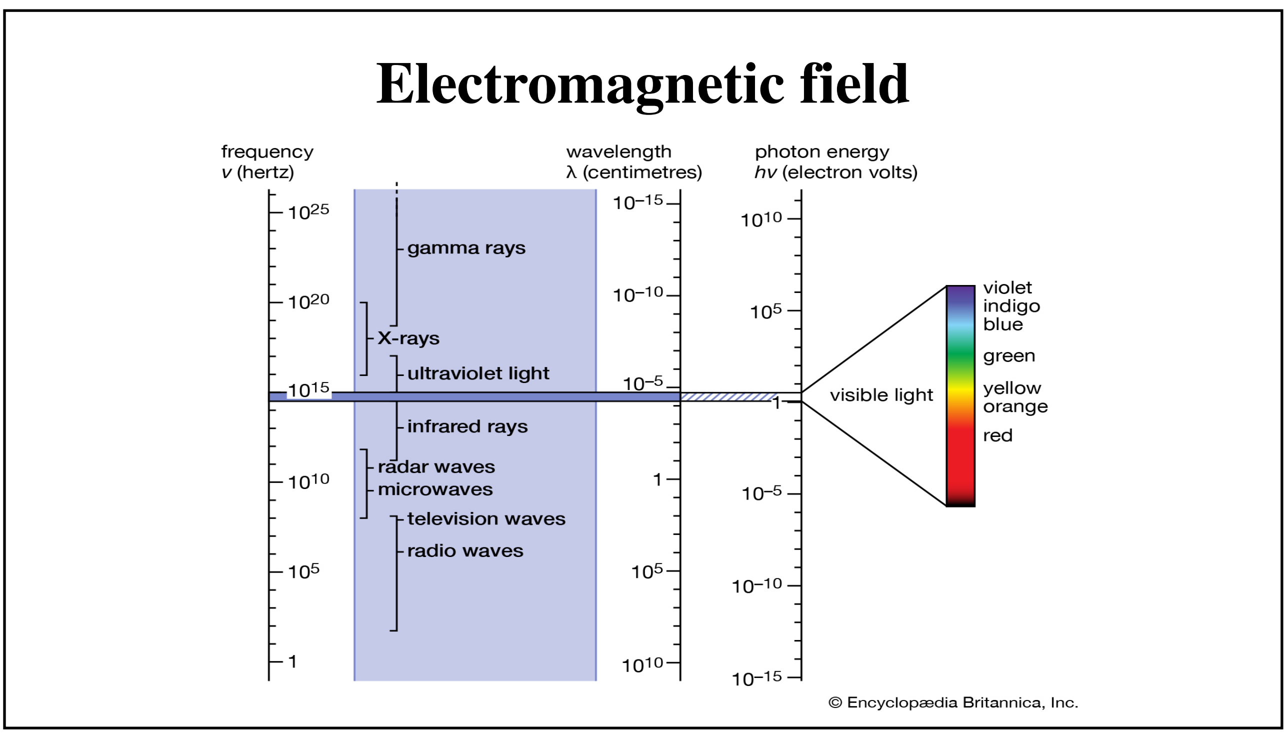
1

Absorbance

- The electromagnetic spectrum consists of regions; ultraviolet UV, visible and infrared IR, etc.
- So, these spectrum are used to study protein structure.
- How can we utilise these spectrum to study protein?
- By studying ttransitions between different electronic states that occur in these regions.
- What is the machine used to measure the absorbance?
- Spectrophotometer.

2



3

What happens after a light beam is passed through a sample?

(Electron) gains an **energy**, become more energetic.
And therefore affects the distribution of electrons in the molecules….

The properties of Light **The properties of matter**

4

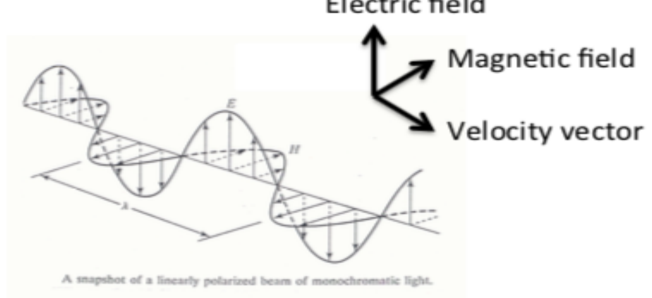
What is The Nature of Light?

- **Electromagnetic radiation:**
- 1- particles
- 2- waves.
- **Properties**

1. Energy $E=hv = hc/\lambda$

when $c= \lambda\ v = 3X10^8\ m.s^{-1}$

1. Wavelength
2. Frequency
3. Magnetic field



- **E= electromagnetic field**
- **H magnetic field**
- **h= Planks constant**
- **Frequency $v=10^{15}\ s^{-1}$**

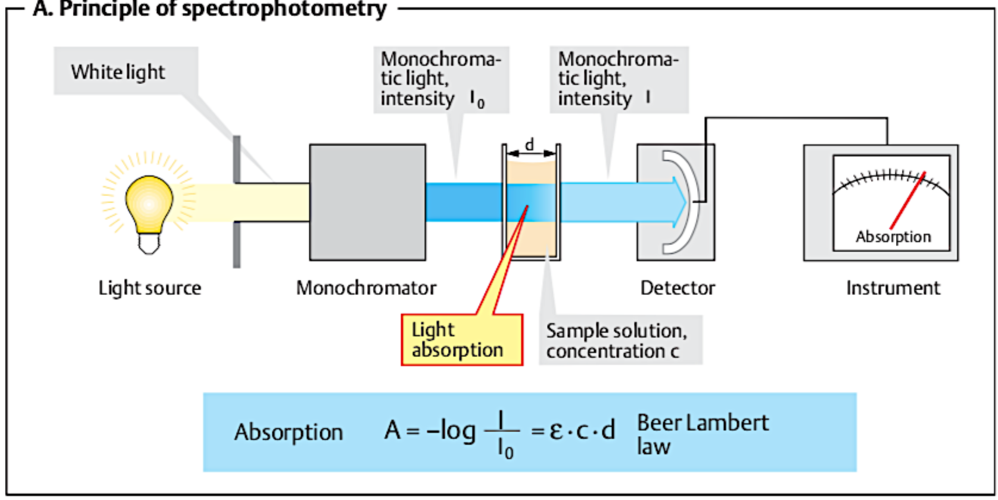
5

What is Absorbance?

- Absorbance is a measure of the quantity of light absorbed by a sample. It is also known as optical density, extinction, or decadic absorbance.
- Absorbance involves transitions of outer shell electrons between various electronic states.
- The absorbance of light excites an electron from the ground state to a higher excited state.
- . Absorbance of light leads to transitions from the lowest ground state to a different of energy levels in the first excited state.

6

Describe the principle of spectrophotometry?



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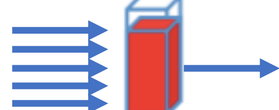
How do you calculate the Absorbance?

- Absorbance is calculated based on either the amount of light reflected or scattered by a sample or by the amount transmitted through a sample.
- If all light passes through a sample, none was absorbed, so the absorbance would be zero and the transmission would be 100%.
- A=1/T

8

Describe Beer-Lambert law?

- The Beer-Lambert law is used to calculate absorbance or conc.:



- **A = ebc**
- Where A is absorbance (no units, $A = log_{10} P_0 / P$)

  e is the molar absorptivity with units of L mol^{-1} cm^{-1}(discussed before).

  b is the path length of the sample, usually the length of a cuvette in centimetres

  c is the concentration of a solute in solution, expressed in mol/L

9

what is a wavelength?

- **Wavelength** is the distance between two repeated peaks or two repeated troughs in a transverse wave.
- **What is the unit of Wavelength?**
- It is usually expressed by the units of nanometres (nm) or micrometres (μm).
- So, What we need to know before measuring A?
- The wavelength of the solution that we are measuring.
- What is the wavelength that is used to measure protein? Why?
- Abs. of proteins are frequently characterized at A280.

10

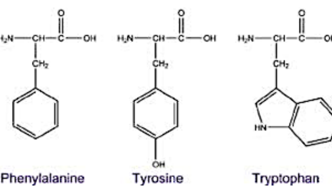
Why proteins absorbed at A280nm?

- Using molecular orbital theory MOT electrons are defined according to the orbitals in which they reside as either σ, π or n (non-bonding) with the corresponding anti- bonding orbitals denoted as σ∗, π∗ or n∗.
- Transitions between σ → σ∗ lie in the far UV region (large energy difference) and are not normally observed by optical methods.
- but transitions between π → π∗ and n → π∗ are frequently observed in the UV and visible region of the electromagnetic spectrum.

11

UV or visible?

- What kind of transitions proteins involve?
- The transitions between π → π∗ and n → π∗ because of the resonance in the aromatics ring.
- What are the amino acids that have aromatic sidechains?
- Tryptophan, Tyrosine and phenylalanine.
- Therefore, proteins show spectra only in the UV region.
- Why proteins do not show spectra at visible region?
- Because there is no electronic transitions appear in visible region (must be colored).



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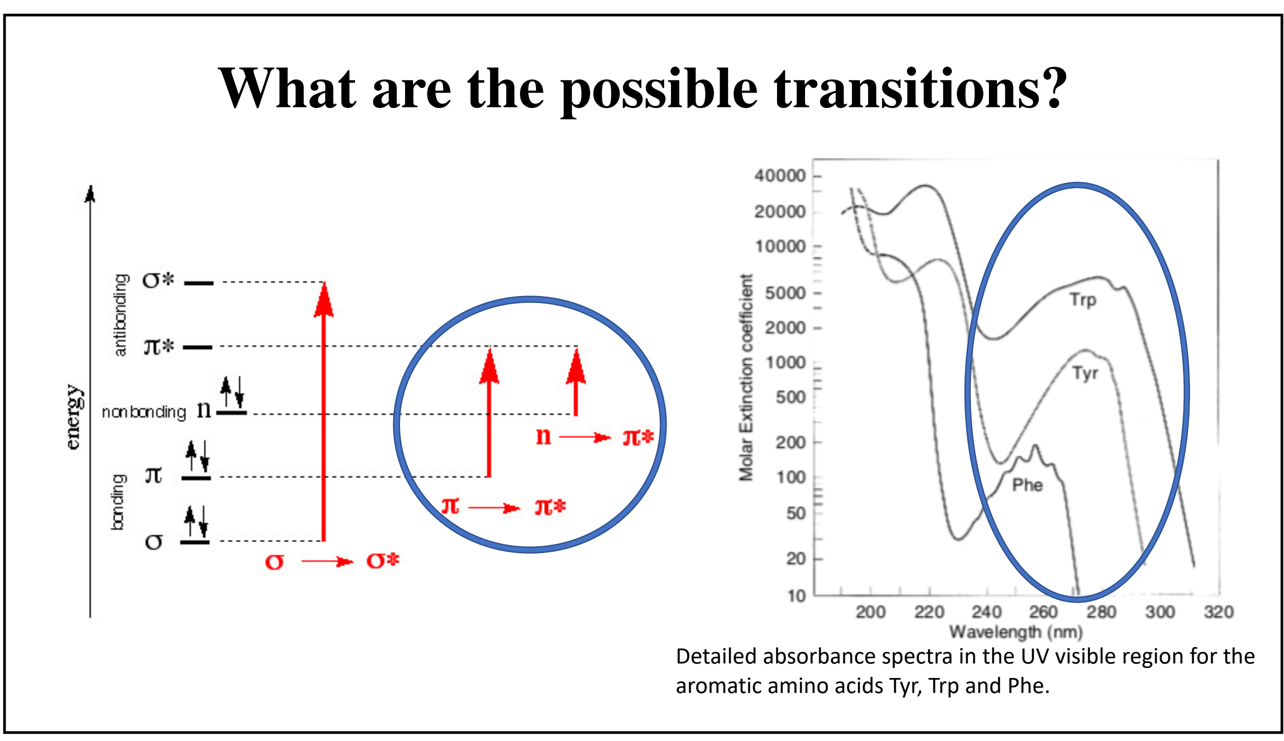
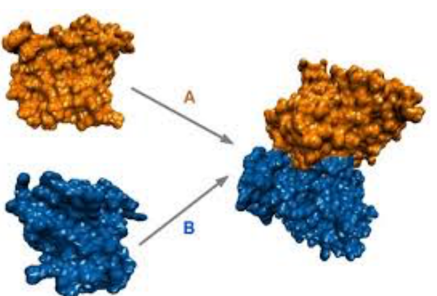


Detailed absorbance spectra in the UV visible region for the aromatic amino acids Tyr, Trp and Phe.

13

What can we use absorbance for?

1. We can use it to measure the concentration of the protein.
2. To study the protein-protein interaction.
3. Drug design.



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How do you measure the protein concentration?

- There are several methods to measure the Conc.
- Measure the Absorption A or called Optical density O.D using spectrophotometer at 280 nm wavelength.
- In this method, we need to know:
- The molar absorptivity E of the protein by calculating the number of aromatic amino acids in the protein which are Tryptophan and Tyrosine (in the websites). Why?
- Because the aromatic ring gives absorption at 280 nm.

15

So, what ?

- So, what does that mean?
- Means: from Beer-lambert equation which is (A=E x b x c) we can calculate the concentration.

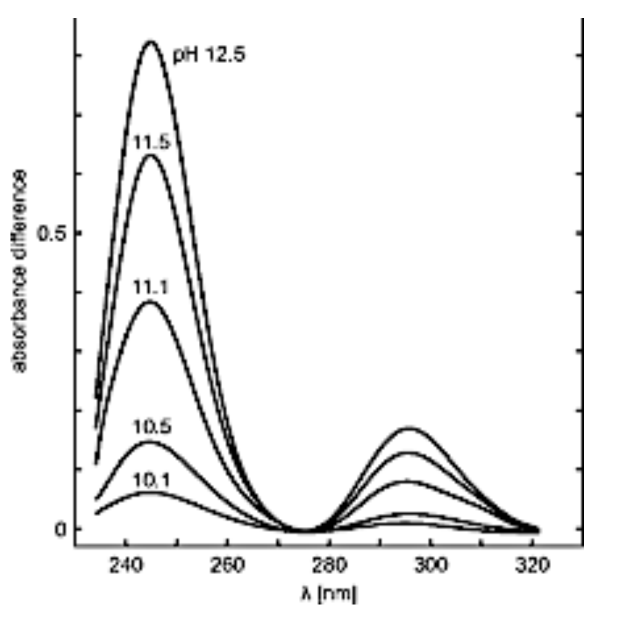
- A= absorption.
- E= molar absorptivity.
- b= cuvette width usually 1 cm.
- C=the concentration

- For example:
- If the molar absorptivity protein = 0.25 g/L
- And we read the O.D (optical density) e.g. A=1
- (A=E x b x c) 1= 0.25 x1 xc
- c=4 mg/ml

16

How do we study the protein-protein interaction by absorbance.

- By following the change in the spectra.
- Then we can fit the data to calculate kd (more datils next lecture).



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Lecture 8

Fluorescence Techniques

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1

What we do study in the biological system?

- We study proteins-protein interactions.
- Proteins are the biomolecules which are units to build the body.
- Proteins as molecules have dynamics.

2

What is fluorescence

- Fluorescence is a phenomenon of the molecule adsorbs of light energy at one wavelength and re-emit it at another, usually longer, wavelength with low energy.
- Some molecules fluoresce naturally.
- Others can be modified to make it fluoresce.
- Fluorescence compounds have two characteristic spectra:

1. An excitation spectrum: wavelength and amount of light absorbed.
2. An emission spectrum: wavelength and amount of light emitted.

- The spectra are the signature or fingerprint of the compounds.
- There is No two compounds have the same fluorescence signature.

3

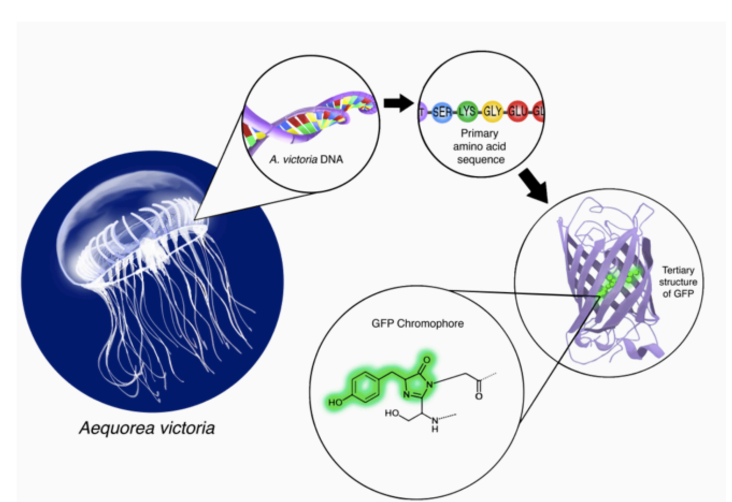
Does fluorescence occur in nature? How?

- Fluorescence occurs in nature as in jellyfish.
- This type of fishes have proteins responsible for fluorescence.
- It is called GFP.

4

Jelly Fish and Green Fluorescent Protein GFP

- What makes jelly fish fluoresce?
- A protein found in the fish which genetically involved in the sequence called GFP.
- What happed when scientists replaced the DNA code of florescence?
- No more florescence showed.



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Biophysical Instrumentation

- What is the measurement of the fluorescence?
- Fluorometry the measurement of the fluorescence.
- What is the instrument used to measure fluorescence?
- Fluorimeter is the instrument used to measure fluorescence.
- Explain how?
- The fluorimeter generates the wavelength of the light that is required to excite the analyte of interest.
- It then transmits the wavelength of light emitted; and then measures the intensity of the emitted light.

6

What are Fluorophore and chromophore?

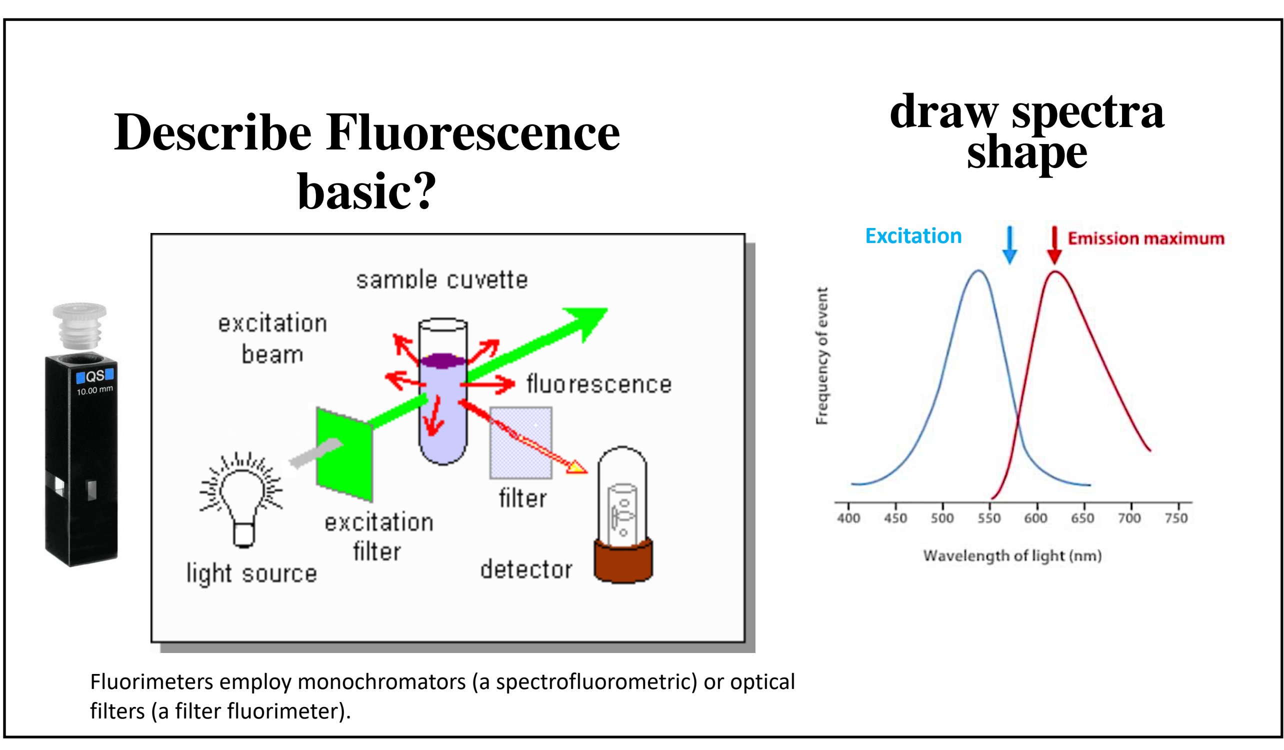
- **The chromophore** is (chemistry) that part **of** the molecule **of** a dye responsible **for** its colour while …
- **The fluorophore** is (biochemistry) a molecule or functional group which is capable **of** fluorescence.
- So, not every **chromophore is fluorophore but ..**
- **Each fluorophore is chromophore.**

7

Describe the Fluorescence mechanism?

- When a photon of excitation light is absorbed by an electron of a fluorescent particle called fluorophores or simply Flours (low energy), which increases the energy level of the electron to an excited state.
- The energy is emitted as a photon to bring the electron back to its ground state (in a single step).
- This emission show fluorescence.

8



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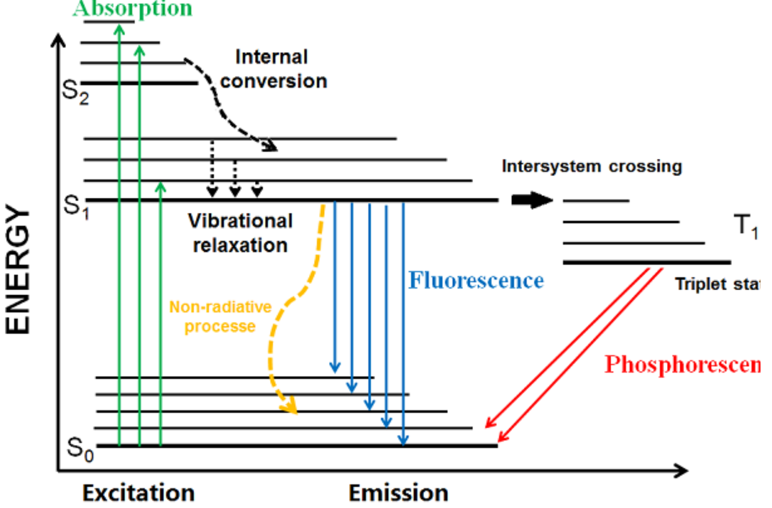
Which has longer wavelength?

- Why the emitted fluorescence can be distinguished from the excitation light?
- The emitted photon usually carries less energy and therefore has a longer wavelength than the excitation photon.

10

Describe Fluorescence mechanism? Jablonski diagram



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Jablonski diagram: 3-stages process:

- **So, Fluorescence is the results of 3-stages process:**
- Excitation of fluorophore due to the absorption of light energy.
- Transient of light exciting time with loss of some energy (very short 10^{-9} -10^{-15} sec).
- Return the fluorophore to the ground state with an emission of light.

12

What are the experimentational artifacts of Florescence?

- Photo-bleaching:
- It occurs when a fluorophore permanently loses the ability to fluoresce due to photo-induced chemical damage or modification.
- Quenching:
- Process leads to reduce the fluorescence intensity or the quantum yield.
- Wrong concentrations

13

How can we use fluorescence in biology

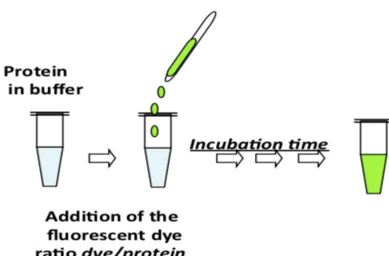
- There too many applications for the fluorescence in biology: e.g

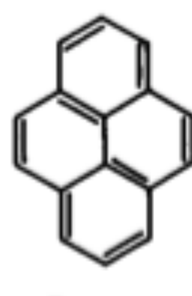
1. DNA & RNA sequencing.
2. To measure the conformational change upon the protein-protein interactions.
3. Enzymatic assays
4. Microscopy
5. **Cool fluorescent(biosensors.**) e.g: Food contamination etc.
6. **Diagnostic in medicine: Fluorescence imaging in cancer detections.**

14

How do we use florescence to study protein?

- Fluorescence is used for protein-protein interactions.
- Protein-protein interactions could be measured by following the conformational change due to the binding.
- We need to label the protein by a fluorophore (a dye)





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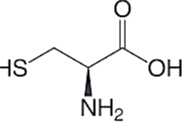
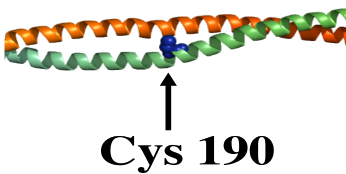
How can we label the protein by fluorophore dye?

- Find the site that dye can attach to it.
- example: a side chain of cysteine in the protein has -SH group.
- But it is found S-S in the protein?
- Unfold the protein by urea.
- React the dye with -SH= S+dye.
- Refold the protein.





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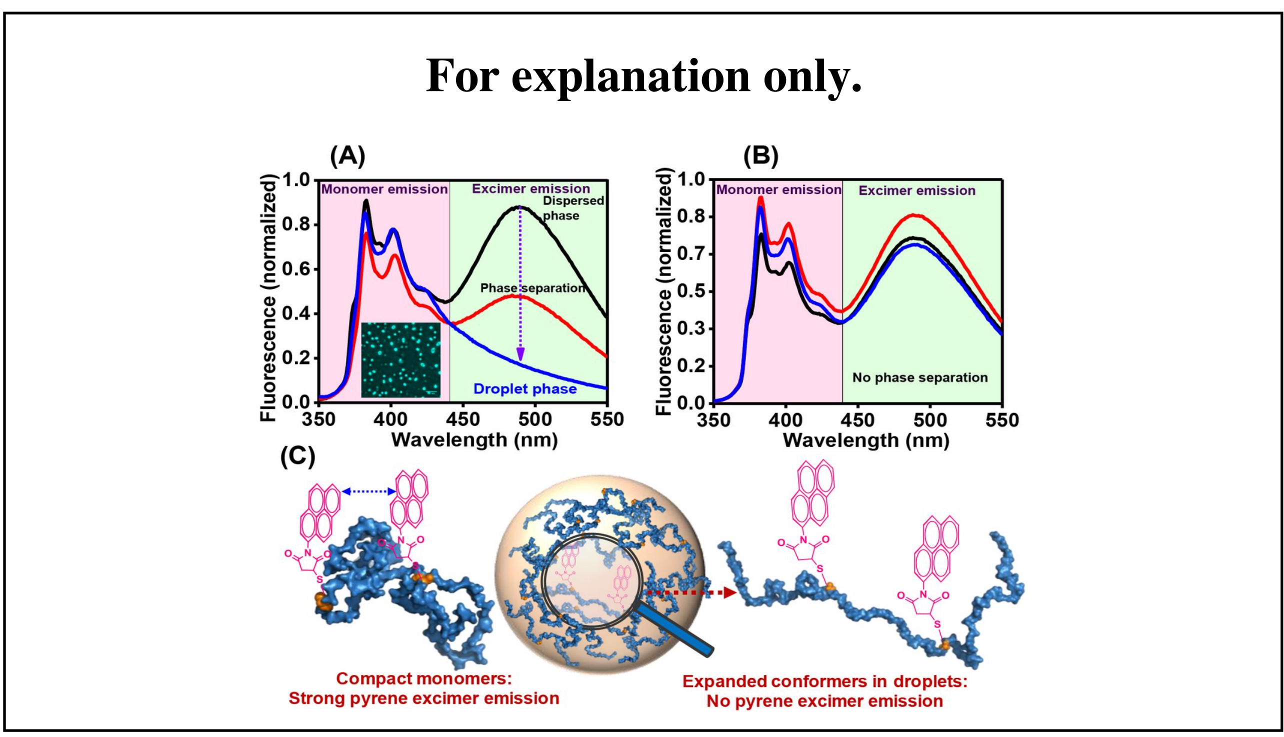
For explanation only.



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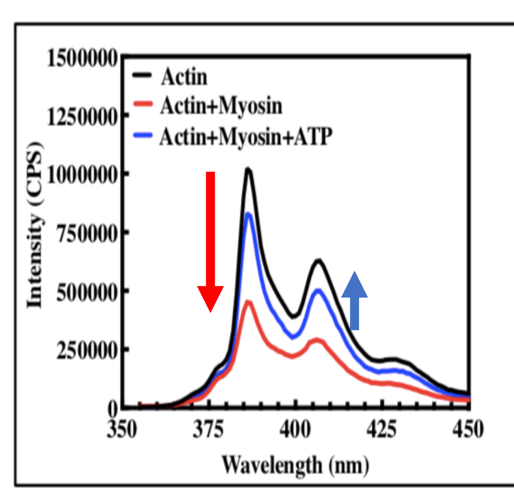
Then we do an assay to detect the binding

- The change in the spectra is upon adding another protein.
- The change in the spectra is due a conformational change in the structure.



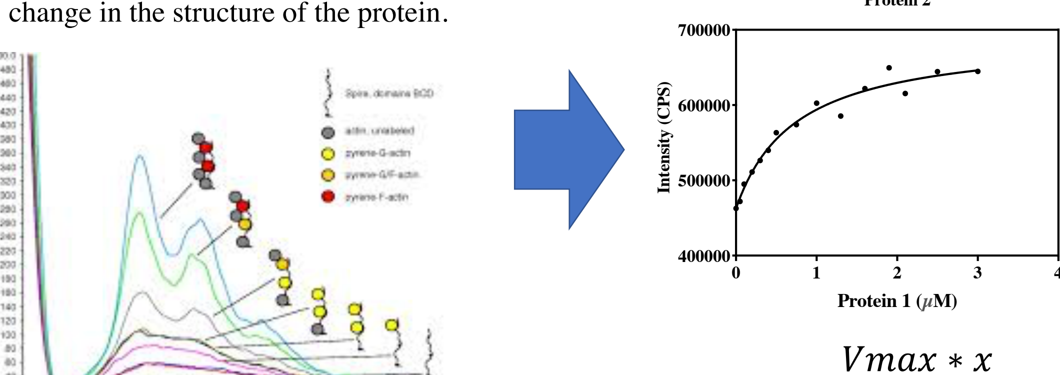
4 The emission spectrum of PIA-actin alone and upon addition of myosin heads and ATP.

18

How can we do protein- protein titration?

1. By following measuring the change of florescence due to adding another protein.
2. The change result form the conformation change in the structure of the protein.

Then we fit the points to calculate Kd (dissociation constant)

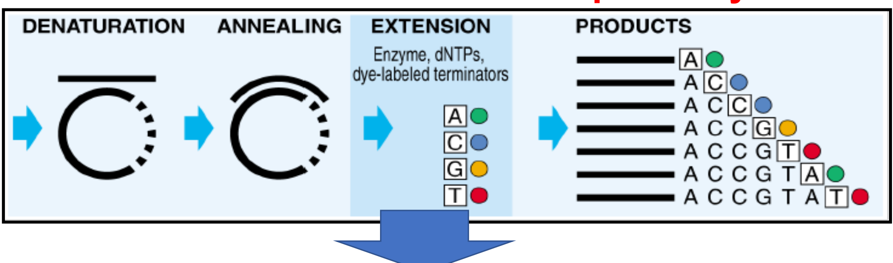


$$y = \frac{Vmax * x}{Kd + X}$$

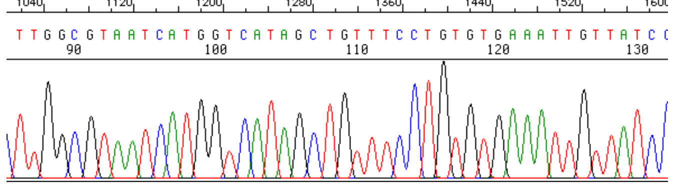
19

How do we use Fluorescence for DNA & RNA sequencing?

Labelling the Terminator Sequencing of each nucleotide with a fluorophore dye



Then we check the sequence of DNA by software…



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Lecture 9

Kinetics study of proteins

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1

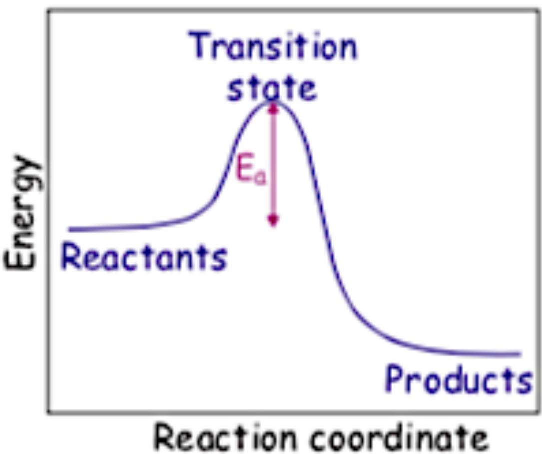
Why do we study kinetics for the biological system

- We study the speed of the 2 proteins ‘enzymes’ interact to produce a movement.
- Proteins as molecules have kinetics and different reaction rates.
- So, therefore, we study the kinetics of the proteins by measuring the speed of the reactions during the interactions.
- How? What are based on?
- It is based on the formation of Product or disappearance of reactants.

2

What information do we get from kinetics?

- All involve a transition between reactants s R and products P.
- Measuring the rate of the reaction and the equilibrium between reactants and products.



3

What is Rate (speed)?

- **What is rate, rate constant?**
- In physics:
- Rate= distance/ time. So, the rate can be:
- Instantaneous rate: a change of concentration at any particular time.
- Average rate: a change in measured concentration in any particular time.

4

Rate law

- The rate is proportional to concentrations.
- Example: The rate law is v = k[A][B] where each reactant is raised to the first power.
- The coefficient k is called the rate constant.

5

Reaction velocities

- The law of mass action: the rate is proportional to concentration.
- **k is the rate of constant.**
- $\frac{\Delta[A]}{\Delta t} = \frac{d[A]}{dt}$. Therefore, rate of a change A= -k [A_0].
- $\frac{d[P]}{dt} = -\frac{d[A]}{dt} = kt$.
- To drive the equation of the first order:

6

Integration of rate of reaction

$$A + \text{catalyst} \xrightarrow{k} P + \text{catalyst}$$

$$\text{Rate} = v = \frac{d[P]}{dt} = \frac{-d[A]}{dt} = k$$

$$Rate = \frac{-d[A]}{dt} = k[A]$$

$$\frac{-d[A]}{dt} = k[A]$$

$$\frac{-d[A]}{[A]} = kdt$$

$$\int_{A_0}^{A_t} \frac{-d[A]}{[A]} = \int_0^t kdt$$

$$\int_{A_0}^{A_t} -d\ln[A] = kt$$

$$\ln[A_0] - \ln[A_t] = kt$$

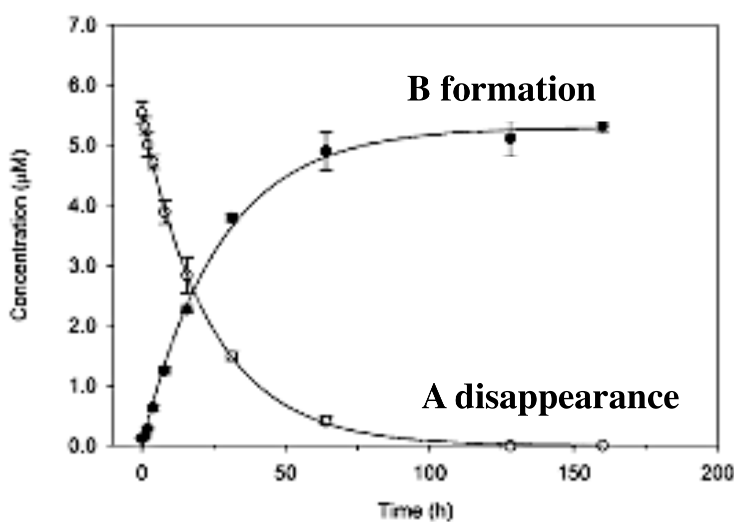
$$\ln[A_t] = -kt + \ln[A_0]$$

For exponential fitting we can use:

$$A = A_0\, e^{-k1t}$$

7

What happened with reaction progress?
Formation of Product or disappearance of reactants



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Describe the First order reaction?

- Rate of reaction depends on the concentration of reactants (not linear).
- As the reaction processed, the concentration changes, and the reaction becomes slower.
- The reaction it is irreversible.
- We can monitor the disappearance of the [A] or the formation of [B] as a function of time.
- But, practically, we want to determine the rate constant of the product formation.

9

From curve to linear

First order (simple)

mono-exponential decrease

slope = $k_{obs} = k_1$

$k_{obs} = k_1$

10

What is Half-life?

Half of life is a time scale, on which the initial concentration that is decreased by half ½ of its original value.

$$\ln[A] = -kt + \ln[A]_0$$

$$\ln\frac{[A]_0}{2} = -kt + \ln[A]_0$$

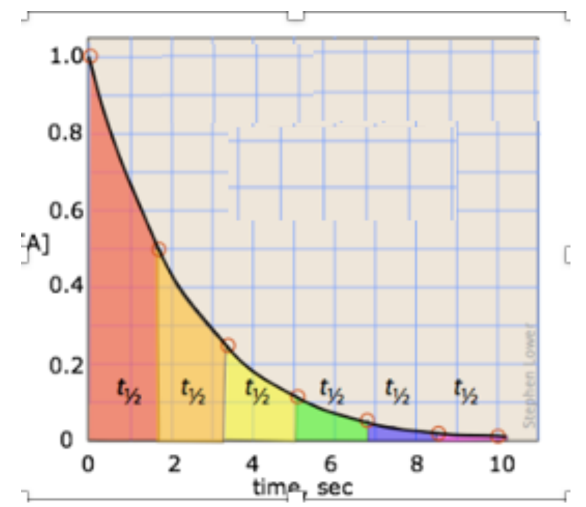
$$\ln[A]_0 - \ln 2 = -kt + \ln[A]_0$$

$$\ln[A]_0 - \ln[A]_0 - \ln 2 = -kt$$

$$-\ln 2 = -kt$$

$$\ln 2 = kt$$

$$t_{1/2} = \frac{\ln 2}{k}$$



11

Units of the rate constant

- The units of rate constant are always such that they convert into a rate expressed as concentration divided by time.

$$\text{Rate} = k\,[A]\,[B]$$

$$(\text{mol L}^{-1}\,\text{s}^{-1}) = (\text{L mol}^{-1}\,\text{s}^{-1})(\text{mol L}^{-1})(\text{mol L}^{-1})$$

12

Which reaction order we do use for biochemical reactions?: Pseudo-first order

- **Pseudo-first order: or Semi-second order.**
- In the case, one of the reactants is much larger than other. So, the reaction is irreversible.
- Therefore: **A + B ⟶ P**
- **where B is very large.**
- And therefore**: $K_{obs}= k_1$.**

K_{obs} the observed K

13

So, we measure first K1 and K-1

- We use Pseudo-first order or called semi second order
- Since the 2nd order reaction depends on both reactants, however, if the concentration of one reactant is in large excess over the other one (the reaction is considered as pseudo 1st or semi 2nd):
- **when [B] >> [A], therefore [B] is almost constant.**
- **A + B ⟶ AB**
- **$A=A^{\circ} * e^{-kobs\ t} = A=A^{\circ} * e^{(-K+1 *[B] *t)}$**

14

What do we measure ?

- We measure the amounts of product over time and then we plot and fit the data with equations: **$A=A^{\circ} * e^{-kt}$ which comes from an exponential model: $y=A*e^{Bx.}$**
- Thus, for **$A=A^{\circ} * e^{-kt}$, k_{obs} will be used; $A=A^{\circ} * e^{-kobs * t}$**

$$A \underset{K_{-1}}{\overset{K_{+1}}{\rightleftharpoons}} B \qquad K_{obs}=K_{+1}+K_{-1} \qquad \text{But.} \quad K_{eq}=K_{+1}/K_{-1}$$

$$= 1 + 2 = 3\ S^{-1}$$

15

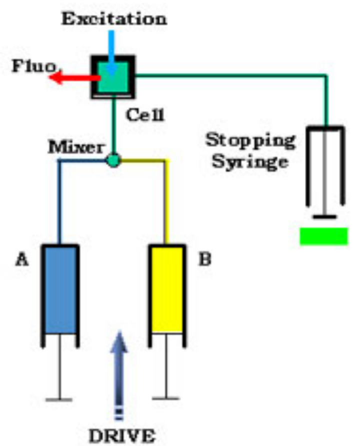
We also detect the association and the dissociation from rate constant

- **Also,**
- **K_{+1} is k Forward k_f**
- **K_{-1} is k reverse k_r**
- **$K_d = K_r/ k_f$ ....dissociation of the product.**
- **$K_a = K_f/ k_r$ ...... association of the product.**
- **<u>NOTES:</u>**
- Measuring the association and the dissociation is very informative.

16

What is Stopped flow machine ?

- Stopped flow instrument is a very rapid mixing device used to study chemical kinetics of fast reactions in solution.
- The reactants are monitored by observing the change in absorbance or (Fluorescence) of the reaction as a function of time (it measures UV/vis in the same time).



17

Approach to equilibrium

- **Equilibrium:** It is a condition in which all influences actions cancel each other. $aA + bB \rightleftharpoons cC + dD$
- Also, it is a state in which both reactants and products are present in concentrations which have no further tendency to change with time.
- The rate of forward process= rate of reverse process.
- **The equilibrium constant Keq** $K = \frac{[C]^c[D]^d}{[A]^a[B]^b}$ $\frac{\text{products}}{\text{reactants}}$
- It is the ratio between amounts of reactants and the products for particular chemical reaction used to calculate behaver.

18

What is the relationship between K equlibrium and kobs?

- $\boldsymbol{Keq = \frac{kf}{kr} \; or \; k\frac{forward}{kreverse}}$ **or = k_{+1}/k_{-1}.**
- **We have measured K_B between blocked and closed states.**
- If KB > 1 that means the product is dominant.
- If KB < 1 that means the reactant is dominant.
- if KB =1 then 50 % of the reactant is dominant.
- **50% = 50%**

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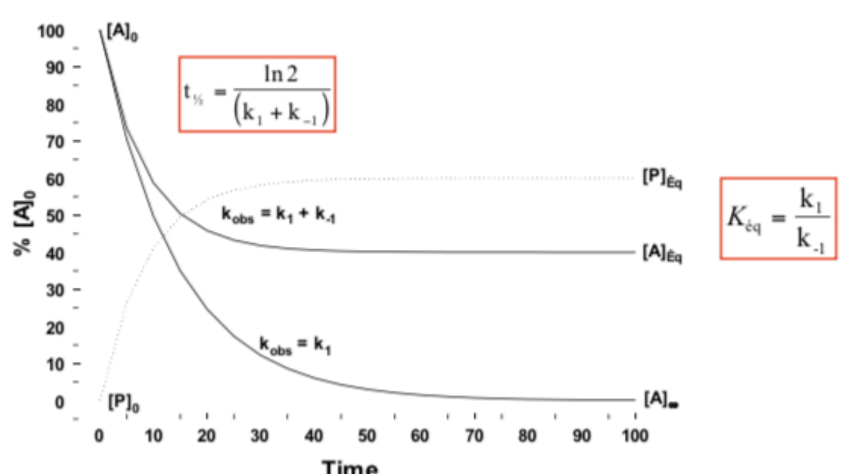
Kinetics and equilibrium



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KB and k obs :Muscle system as example

- Actin + myosin +
- Tropomyosin TPM +Troponin Tn
- We calculate k obs from curves.
- Then we calculate KB from kobs.

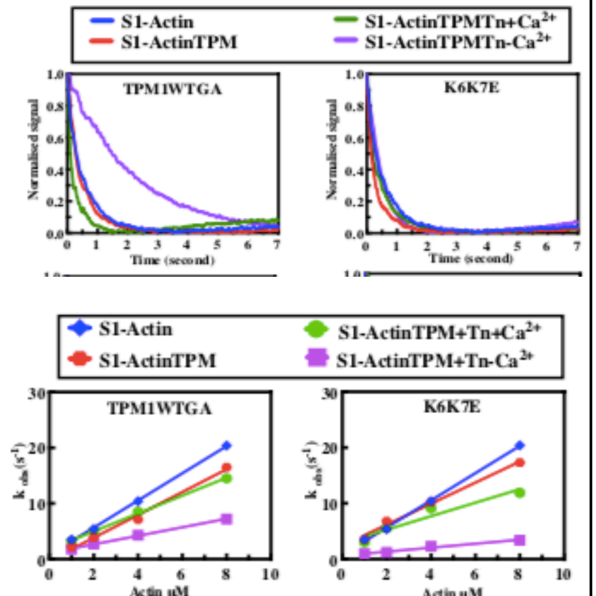

Table 4.5 Effect of TPM mutants on K_B parameter.

The table shows the summary of the calculation of the K_B parameter which represents the equilibrium between blocked and closed stated in the filament regulation in both ($\pm Ca^{2+}$).

TPM	K_B ($+Ca^{2+}$)	K_B ($-Ca^{2+}$)	K_B (TPM)
TPM1WTGA	1.92 ± 0.08	0.59 ± 0.04	3.88 ± 0.15
K6K7E	1.73 ± 0.26	0.21 ± 0.05	2.73 ± 0.14

21

Example for pseudo-order reactions

- If a 2nd order reaction has the rate equation R = k[A][B], and the rate constant, k, is $3.67M^{-1}s^{-1}$, [A] is 4.5M and [B] is 99M, what is the rate constant of its pseudo-1st-order reaction?
- Because [B] is in excess we multiply 99M with $3.67M^{-1}s^{-1}$
- $(99M)(3.67M^{-1}s^{-1}) = 363.33s^{-1}$
- https://chem.libretexts.org/Bookshelves/Physical_and_Theoretical_Chemistry_Textbook_Maps/Supplemental_Modules_(Physical_and_Theoretical_Chemistry)/Kinetics/Reaction_Rates/Second-Order_Reactions/Pseudo-1st-order_reactions

22

units ?

- What are the units of k in A = k[A]? A. mol L^{-1} s^{-1}
- B. L mol-1 s^{-1}
- C. mol L^{-1}
- D. s^{-1}

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Question ?

- A first order process occurs with a rate constant of 0.1 s^{-1}. If the initial concentration $[A]_0$ = 1 M what is the concentration after 30 seconds? A. 0.5 M
- B. 0.05 M
- C. 0.005 M
- D. 0.0005 M

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Lecture 10

Circular dichroism CD

a physical instrument for secondary structure check

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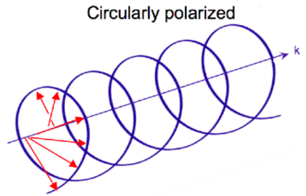
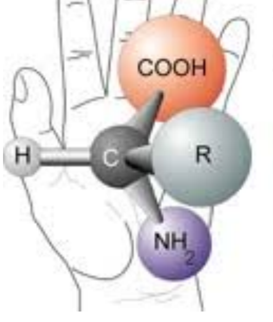
1

What is a Circular dichroism ?

- Circular dichroism is a spectrophotometer observes differences in absorption of right and left circularly light by chiral molecules (of protein). https://www.youtube.com/watch?v=Fu-aYnRkUgg
- $\Delta E = E_R - E_L$.
- It the protein molecules are chiral? How?





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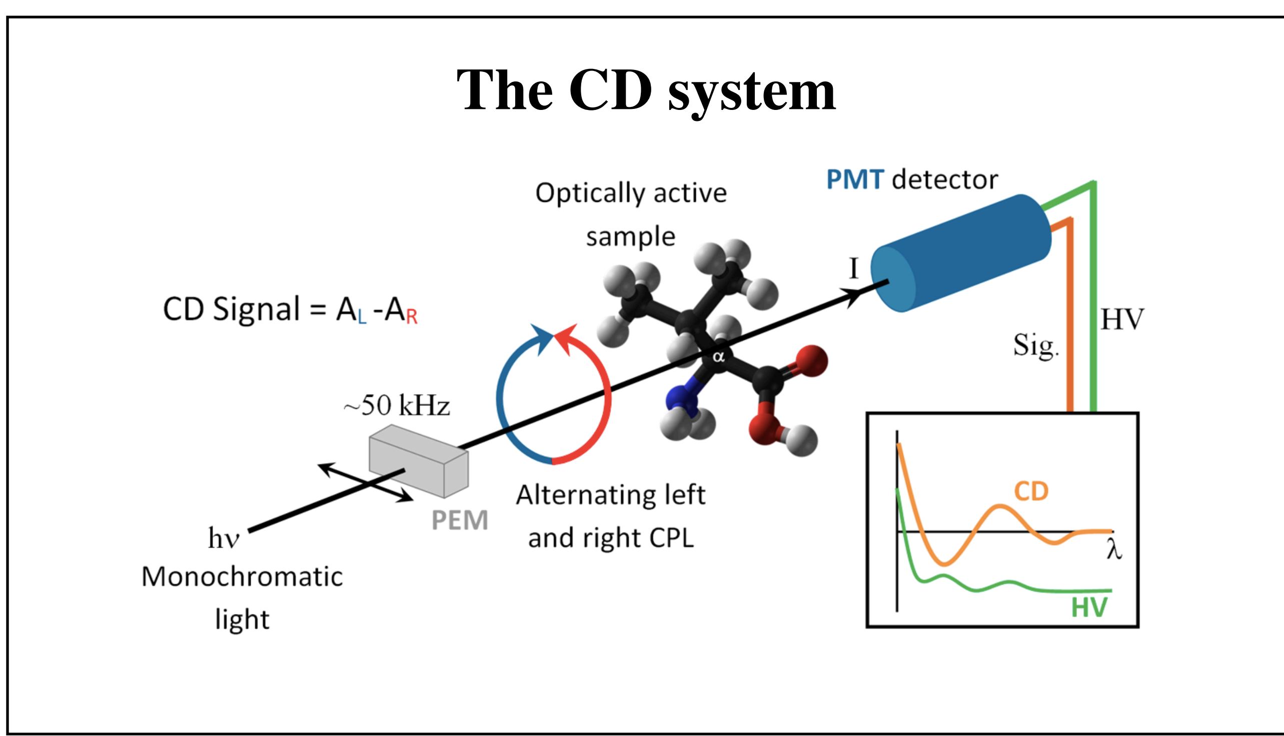
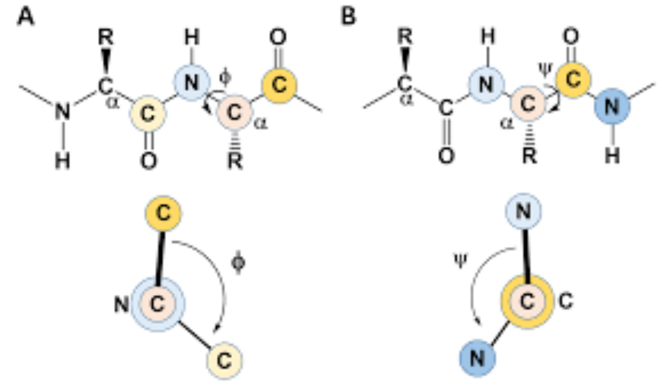
The CD system



3

How many two types of dihedral angles for peptides that can rotate the polarized light?:

- There are two types of dihedral angles for peptides that can rotate the polarized light:
- Ψ Psi is between C-Cα
- θ Phi is between Cα-N



4

What is the Circular dichroism used for?

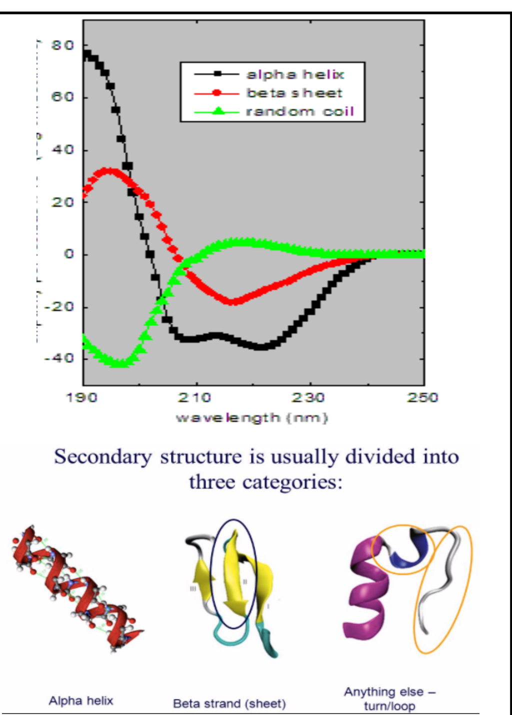
CD spectroscopy is mainly used to:

1. To determine the secondary structure of proteins.
2. To determine whether an expressed, purified protein is folded or not.
3. To determine if a mutation affects its conformation or stability.
4. It can be used to study protein interactions.

5

How does CD show the spectra? Explain?

- CD determine the type of the secondary structure of the protein as shown in the figure:
- Also, to to estimate the percentage of the helixes, beta sheet and random coil.



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Usually, we compare the CD spectra of the WT and the mutants.

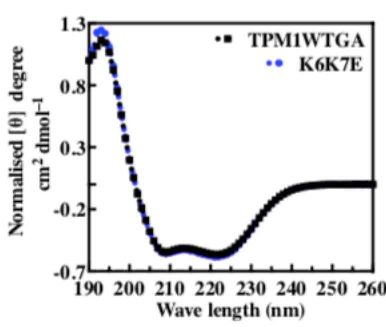


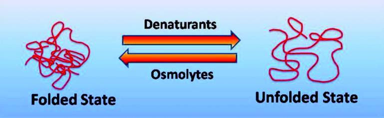
Table 4.1 α-helical calculation and beta strands in the TPM secondary structure (195-260 nm).

TPM	α Helix %	β Sheet (Parallel and anti-parallel) %	β bends %	Random coil %
TPM1WTGA	99.45 ± 0.1	0.65 ± 0.1	1.7 ± 0	1.8 ± 0.1
K6K7E	98.7 ± 1.6	0.75 ± 0.1	1.7 ± 0	1.65 ± 0.1

7

How does CD signal change with Temperature?

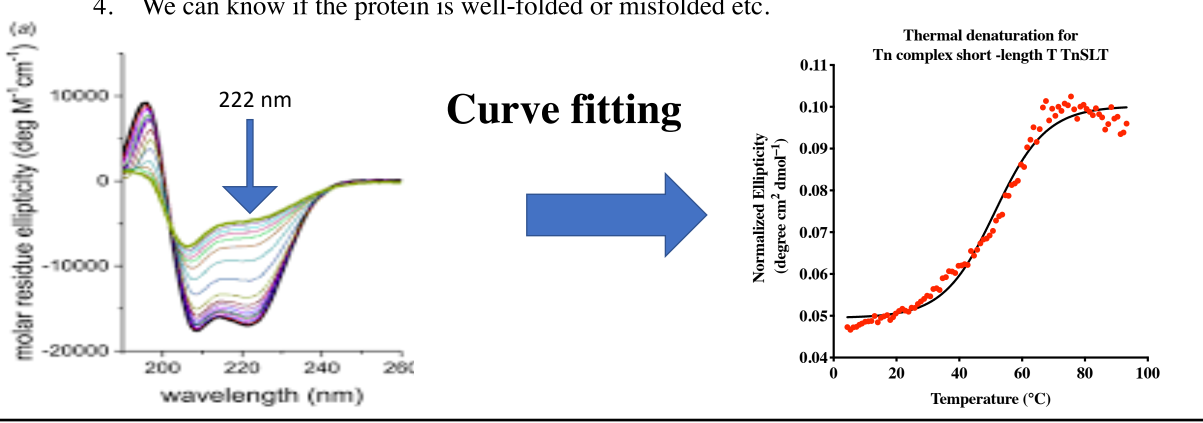
- With Temperature increases, the protein begins to unfold till becomes totally unfolded.
- So, at this point we can collect the points of the spectra and fit it as an exponential curve to calculate Tm˚C (mid point).
- What is Tm.˚C?
- The mid-point is the point between folded and unfolded state.



8

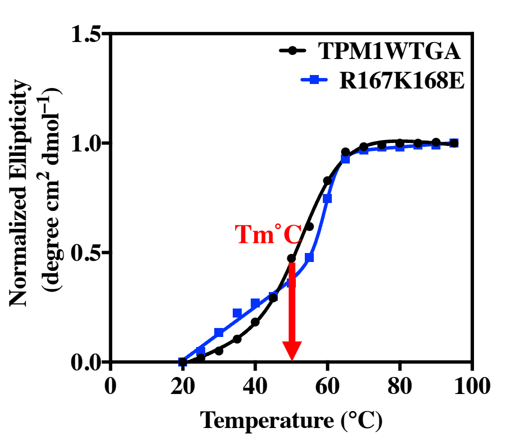
Describe the change in the CD spectra with T ˚C increases

1. The point are collected at 222 nm and then fitted as an exponential curve.
2. The mid-point is calculated.
3. Then we compare the mid point of the WT with mutants.
4. We can know if the protein is well-folded or misfolded etc.



Protein folding and unfolding

- Protein **Misfolding** could cause Disease.
- It can be studies by measuring Tm ˚C (mid temperature degree)using CD (thermal unfolding) technique.
- It shows a sigmodal curve.



Lecture 10

Circular dichroism CD Binding assay (two different topics)

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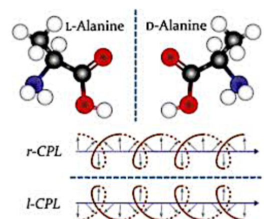
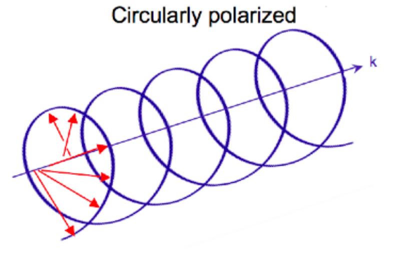
1

What is a Circular dichroism ?

- Circular dichroism is a spectrophotometer observes differences in absorption of right and left circularly light by chiral molecules (of protein).
- $\Delta E = E_R - E_L.$
- It the protein molecules are chiral? How?





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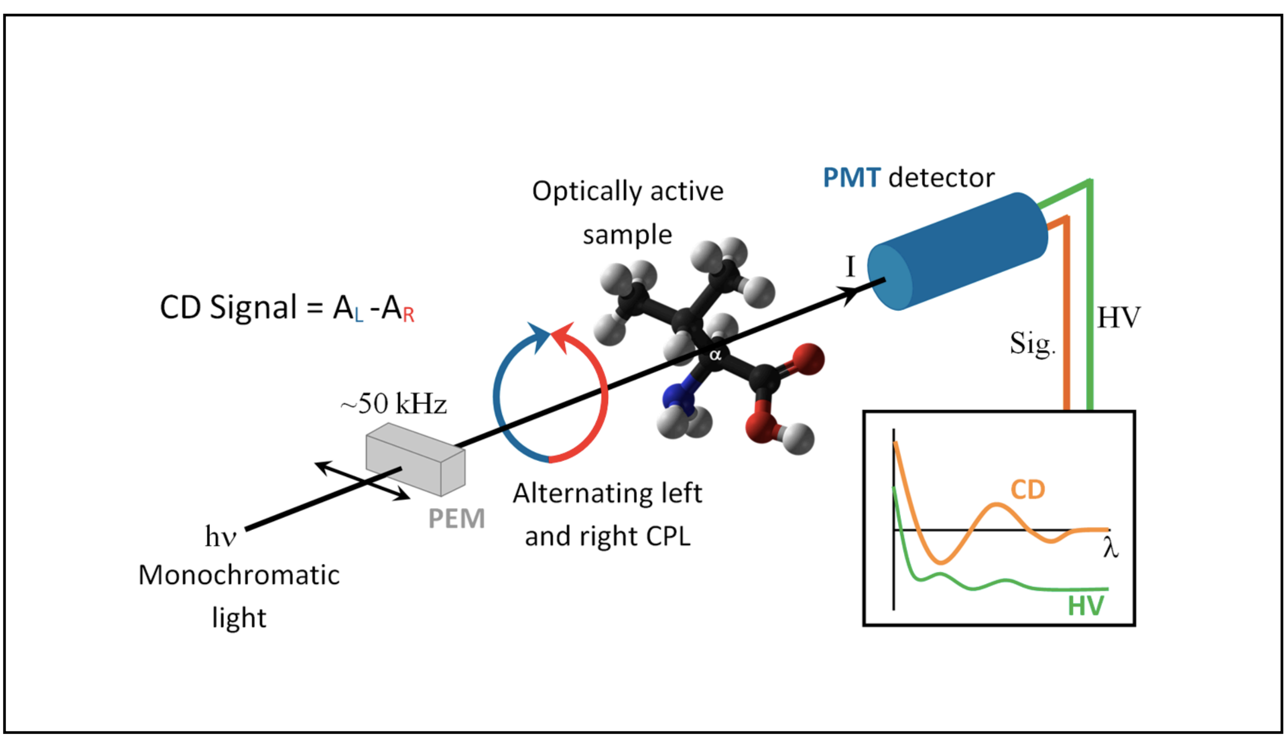

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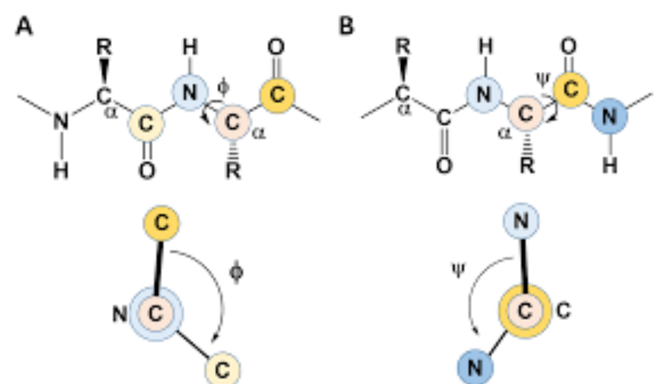
How many two types of dihedral angles for peptides that can rotate the polarized light?:

- There are two types of dihedral angles for peptides that can rotate the polarized light:
- Ψ Psi is between C-Cα
- Θ Phi is between Cα-N



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What is the Circular dichroism used for?

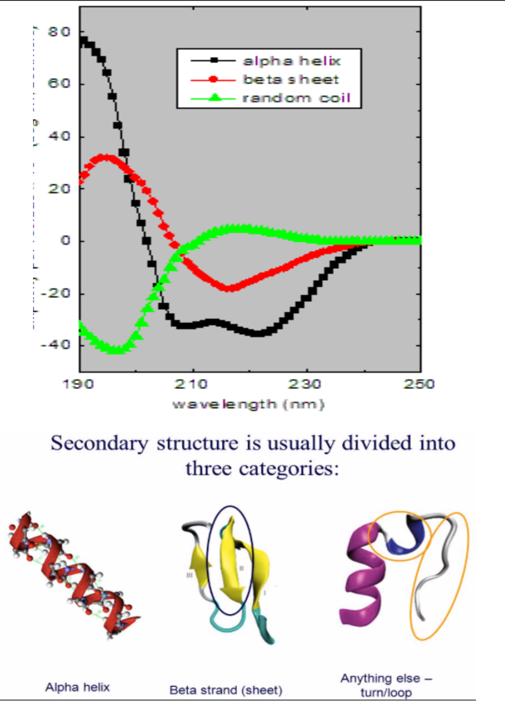
CD spectroscopy is mainly used to:

1. To determine whether an expressed, purified protein is folded or not.
2. To determine the secondary structure of proteins.
3. To determine if a mutation affects its conformation or stability.
4. It can be used to study protein interactions.

5

How does CD spec. show the spectra? Explain?

- CD determine the type of the secondary structure of the protein as shown:
- Also to to estimate the percentage of the helixes, beta sheet and random coil.



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Usually we compare the CD spectra of the WT and the mutants.

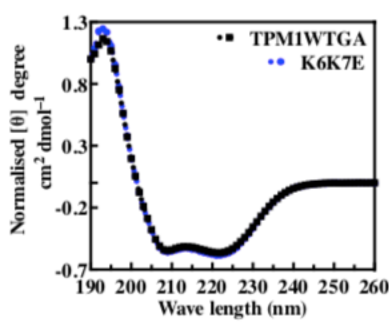


Table 4.1 α-helical calculation and beta strands in the TPM secondary structure (195-260 nm).

TPM	α Helix %	β Sheet (Parallel and anti-parallel) %	β bends %	Random coil %
TPM1WTGA	99.45 ± 0.1	0.65 ± 0.1	1.7 ± 0	1.8 ± 0.1
K6K7E	98.7 ± 1.6	0.75 ± 0.1	1.7 ± 0	1.65 ± 0.1

7

How does CD signal change with Temperature?

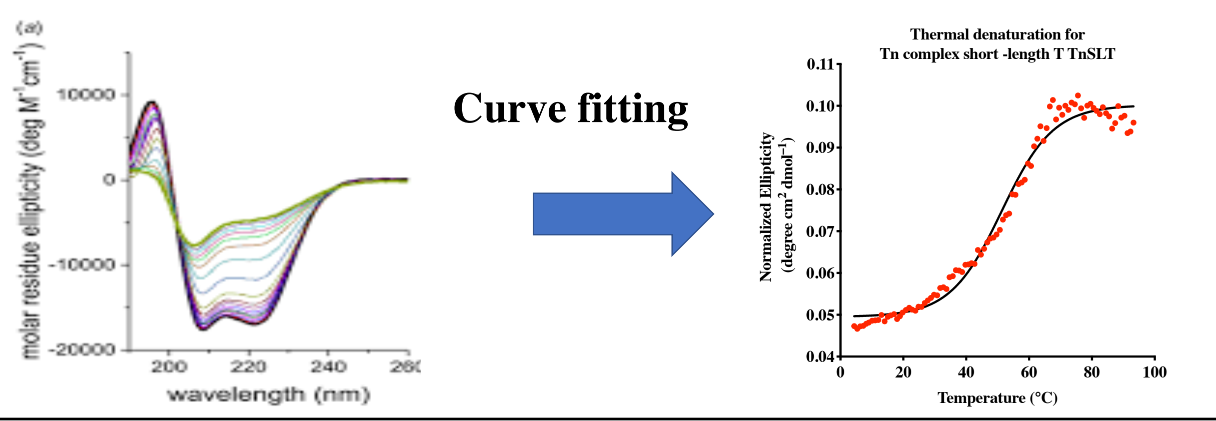
- With Temperature increases, the protein begins to unfold till becomes totally unfolded.
- So, at this point we can collect the points of the spectra and fir it as an exponential curve to calculate Tm˚C.
- What is Tm.˚C?
- It the mid-point between folded and unfolded state.

Denaturants
Osmolytes
Folded State
Unfolded State

8

Describe the change in the CD spectra with T ˚C increases

1. The point are collected at 222 nm and then fitted as an exponential curve.
2. The mid-point is calculated.
3. Then we compare the mid point of the WT with mutants.



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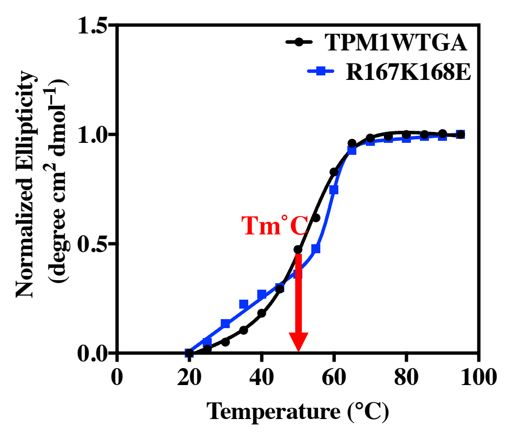
Protein folding and unfolding

- Protein **Misfolding** could cause Disease.
- It can be studies by measuring Tm ˚C (mid temperature degree)using CD (thermal unfolding) technique.
- It shows a sigmodal curve.



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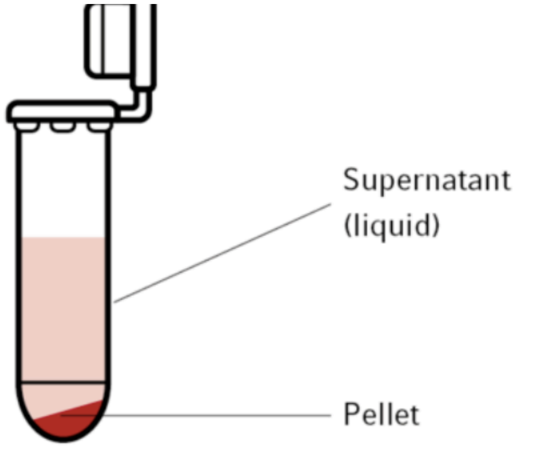
Sedimentation assays ‘’Binding assay”

11

What is the Co-sedimentation assay?

- Co-sedimentation assay is an in vitro assay
- It is done to assess the binding of the 2 proteins or more by sedimentation (centrifugation at high speed about 80,000 rpm).
- The aim of this assay is to examine the ability of filamentous protein such as acin to bind to non- filamentous protein.



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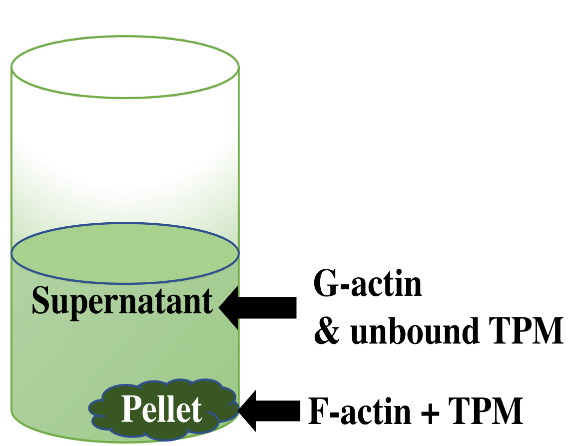
Which protein does sedimented ?

- From history,
- We know that filamentous proteins only sedimented at high speed such as actin.
- However, non- filamentous protein never sedimented such as Tropomyosin Tpm and G-actin (non-filamentous)



13

TPM1WTGA
+Actin -Actin
S P S P M

How to check the binding of the Co- sedimentation?

- **The samples aare taken from the supernatant and the pellet and then checked by by SDS-page of gel electrophoresis:**
- **If the actin binds to Tropomyosin TPM, it will sedimented in the pellet.**
- **If not, it will remain in the supernatant.**

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Lecture 11

Electron Microscopy

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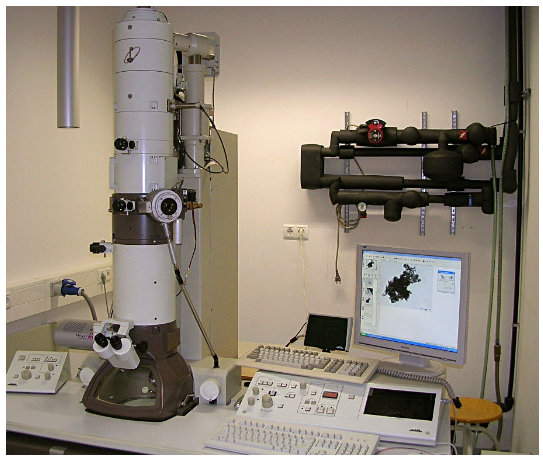
1

Electron Microscopy (EM)

- The are two type of Electron Microscopy:

1. Transmission Electron Microscopy (TEM)
2. Scanning Electron Microscopy (SEM)



2

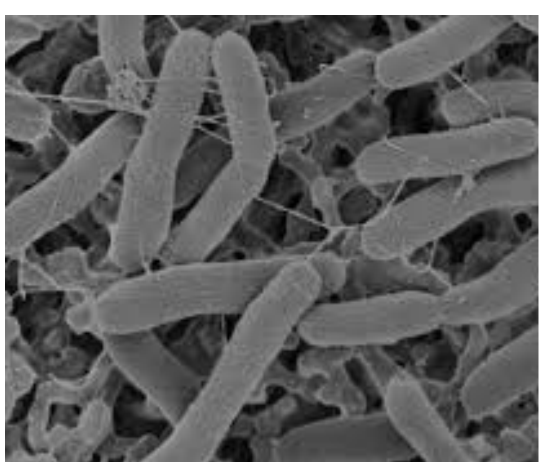
What is TEM?

- The transmission electron microscope is a very powerful tool for material science.
- TEM uses a high energy beam of electrons is shone through a very thin sample, and the interactions between the electrons and the atoms to observe features of a specimen.
- High resolution can be used to analyse the quality, shape, size and density of quantum wells, wires and dots of an image taken.

3

What can TEM reveal ?

- TEMs can reveal the finest details of internal structure - in some cases as small as individual atoms.
- Examples: the crystal structure and features in the structure like dislocations. Etc.



4

What does EM use instead of light? Why?

- The TEM operates on the same basic principles as the light microscope but uses electrons instead of light.
- Because the wavelength of electrons is much smaller than that of light.

5

Describe the imaging?

1. The beam of electrons from the electron gun is focused into a small, thin, coherent beam by the use of the condenser lens.
2. This beam is restricted by the condenser aperture, which excludes high angle electrons.
3. The beam then strikes the specimen and parts of it are transmitted depending upon the thickness and electron transparency of the specimen.
4. This transmitted portion is focused by the objective lens into an image on phosphor screen or charge coupled device (CCD) camera.
5. Optional objective apertures can be used to enhance the contrast by blocking out high-angle diffracted electrons.
6. The image then passed down the column through the intermediate and projector lenses, is enlarged all the way.
7. The image strikes the phosphor screen and light is generated, allowing the user to see the image.

6

Describe the diffraction in the TEM?

1. As the electrons pass through the sample, they are scattered by the electrostatic potential set up by the constituent elements in the specimen.
2. After passing through the specimen they pass through the electromagnetic objective lens which focuses all the electrons scattered from one point of the specimen into one point in the image plane.
3. Also, shown in the figure, a dotted line where the electrons scattered in the same direction by the sample are collected into a single point.
4. This is the back focal plane of the objective lens and is where the diffraction pattern is formed.

7

Imaging and diffraction

Optic axis
Single-crystal specimen
(hkl) plane
A
B
$2\theta_B$
Objective lens
g_{hkl}^*
O*
Diffracted beam
Transmitted beam
Diffraction pattern
Objective aperture
www.globalsino.com/EM/
B'
A'
Bright field image

8

Describe the preparation techniques for TEM?

1. ***Dimpling*** is a preparation technique that produces a specimen with a thinned central area and an outer rim of sufficient thickness to permit ease of handling.
2. ***Ion milling*** is traditionally the final form of specimen preparation. In this process, charged argon ions are accelerated to the specimen surface by the application of high voltage. The ion impingement upon the specimen surface removes material as a result of momentum transfer

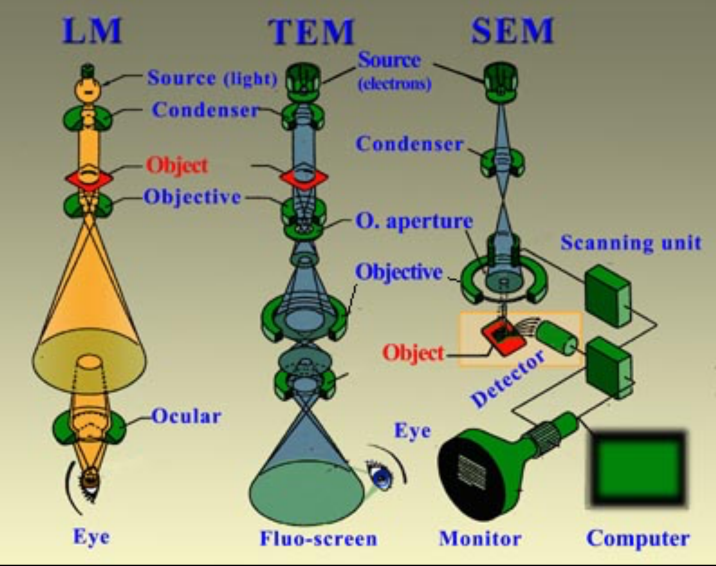
9

What is SEM?

- SEM:
- SEM is used for inspecting topographies of specimens at very high magnifications.
- SEM magnifications can go to more than 300,000 X.

10

The differences in tools of microcopies



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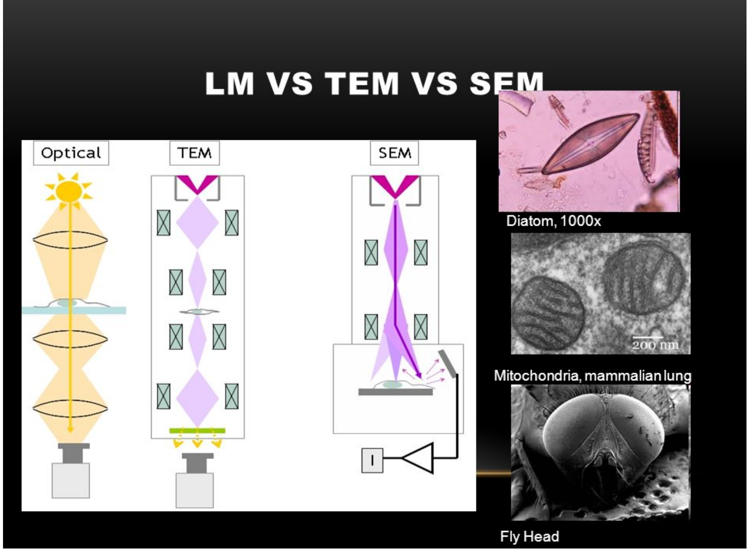
The difference in an image resolutions



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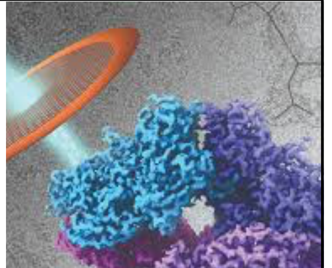
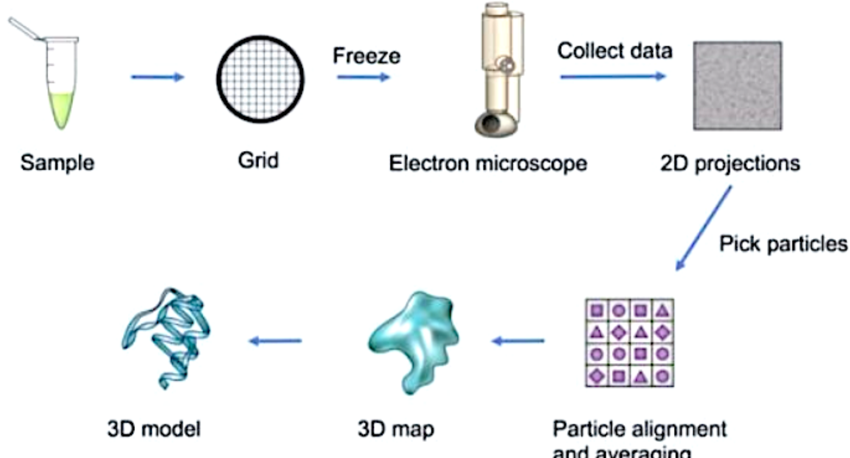
What is Cryo-EM?



- **Cryogenic electron microscopy** (**cryo-EM**) is an electron microscopy (EM) technique applied on samples cooled to cryogenic temperatures (means very low degree temperatures) and embedded in an environment of vitreous water.
- An aqueous sample solution is applied to a grid-mesh and plunge-frozen in liquid ethane.
- While development of the technique began in the 1970s, recent advances in detector technology and software algorithms have allowed for the determination of biomolecular structures at near-atomic resolution.
- This has attracted wide attention to the approach as an alternative to X-ray crystallography or NMR spectroscopy for macromolecular structure determination without the need for crystallization.

13

Cryo EM: from sample to structure ?



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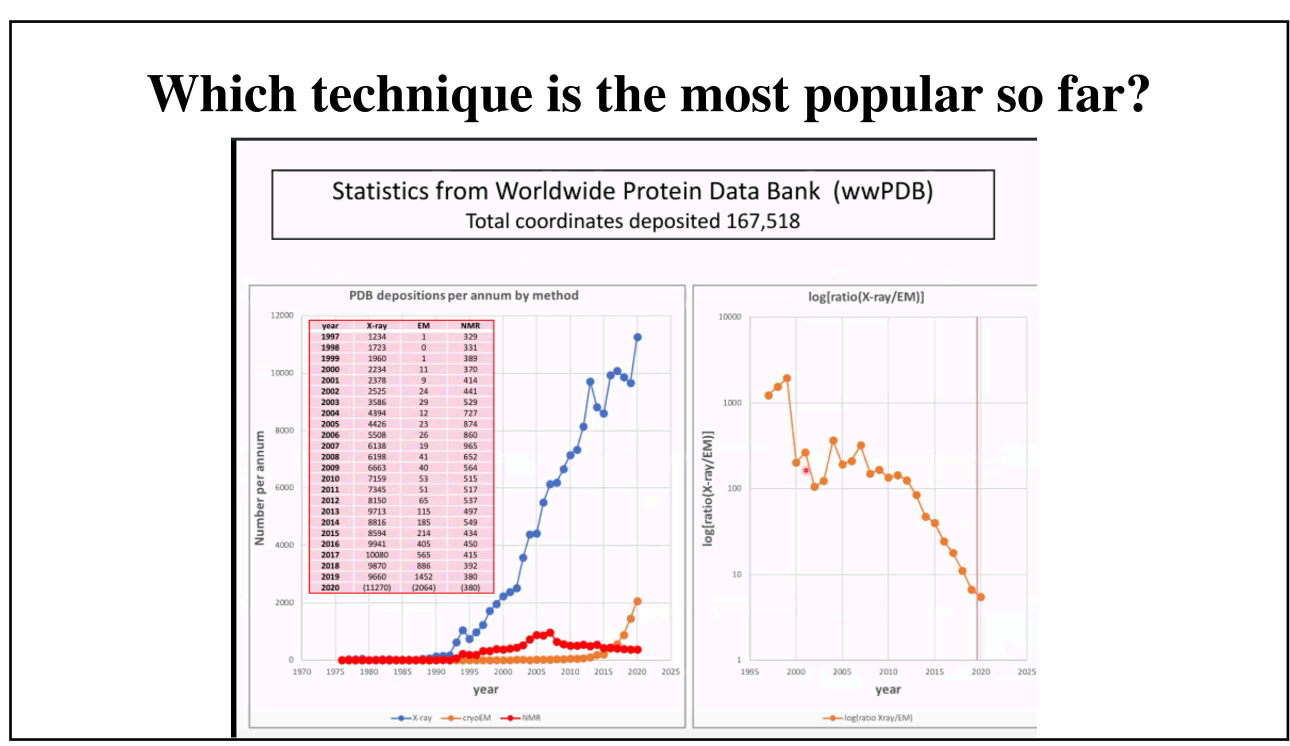

year	X-ray	EM	NMR
1997	1234	1	329
1998	1723	0	331
1999	1960	1	389
2000	2234	11	370
2001	2378	9	414
2002	2525	24	441
2003	3586	29	529
2004	4394	12	727
2005	4426	23	874
2006	5508	26	860
2007	6138	19	965
2008	6198	41	652
2009	6663	40	564
2010	7159	53	515
2011	7345	51	517
2012	8150	65	537
2013	9713	115	497
2014	8816	185	549
2015	8594	214	434
2016	9941	405	450
2017	10080	565	415
2018	9870	886	392
2019	9660	1452	380
2020	(11270)	(2064)	(380)

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Lecture 12

Thermodynamic of proteins

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1

What we study in the biological system

- We study proteins-protein interactions.
- Proteins are the biomolecules is a unit which the body is built of.
- Proteins as molecules have dynamics.
- So, therefore, we study the dynamic of the proteins by measuring the thermodynamic parameters during the interactions.
- What are the thermodynamic parameters? What are based on?

2

What are the thermodynamic parameters? ΔH

- Enthalpy ΔH, a property of a thermodynamic system, is equal to the system's internal energy plus the product of its pressure and volume.
- **(1st law of thermodynamic)**
- In a system enclosed, the heat absorbed or released equals the change in enthalpy

3

1- ΔH

- Any biological system underly to Exothermic and/or endothermic process.
- To understand that:
- The dissolving of NaOH is an exothermic reaction with $\Delta H < 0$
- So, the solution becomes warm and heat is transferred to the surroundings.
- In contrast, the dissolution of $NaNO3$ is an endothermic process,
- with $\Delta H > 0$.
- As a consequence, the solution becomes cold, as heat is drawn from the surroundings.

4

2- ΔS

- Entropy ΔS: it is a property of thermodynamic system. it tells how order or disorder the system is.
- $\Delta S > 0$ (+) more disorder.
- $\Delta S < 0$ (-) increase order.
- **2nd law: Entropy of any isolated system always increases.**

5

Endergonic and exergonic processes

- Processes with $\Delta G < 0$ are termed *exergonic*, processes with $\Delta G > 0$ are *endergonic*.
- With ΔG, the *free energy G*. The general definition of the free energy is
- $G = H - TS$
- *G* is also called the *Gibbs free energy*. Complete differentiation of *G* gives:
- $\Delta G = \Delta H - T\Delta S$
- For reactions: $\Delta Gr = G$ products $- G$ reactants
- The equilibrium is affected by change on these paprmters.

6

Units of the parameters

$$\Delta G = \Delta H - T\Delta S$$
$$\Delta G = RT\ln K_d$$

ΔG- *Gibbs Free Energy, or "available energy"*
ΔH- *Enthalpy change*
T- *Temperature in Kelvin*
ΔS- *Entropy change*
R- *Gas constant, 8.314JK⁻¹mol⁻¹*
K_d- *Dissociation rate*

Standard Free Energy (ΔG^o) and Temperature (T)

(consists of 2 terms) $\Delta G^o = \Delta H^o - T\Delta S^o$ *(on equation sheet)*

free energy (kJ/mol)	enthalpy term (kJ/mol)	entropy term (J/mol·K)
max energy used for *work*	energy transferred as *heat*	energy *dispersed* as disorder

units convert to **kJ**!!!

The **temperature dependence** of free energy comes **from** the **entropy term** ($-T\Delta S^o$).

7

Solutions ! ΔH -92.22 kJ & ΔS= -198.75 J/K what is ΔG?

$$T_K = 25^\circ C + 273.15 = 298.15\ K$$
$$\Delta S^\circ = -198.75\ \cancel{J}/K \times \frac{1\ kJ}{1000\ \cancel{J}} = -0.19875\ kJ/K$$
$$\Delta H^\circ = -92.22\ kJ$$
$$\Delta G^\circ = \Delta H^\circ - T\Delta S^\circ \qquad \text{Plug in } \Delta H^\circ, \Delta S^\circ, \text{ and } T$$
$$\Delta G^\circ = -92.22\ kJ - (298.15\ \cancel{K})(-0.19875\ kJ/\cancel{K})$$
$$\Delta G^\circ = -92.22\ kJ + 59.257\ kJ$$
$$\boxed{\Delta G^\circ = -32.96\ kJ}$$

8

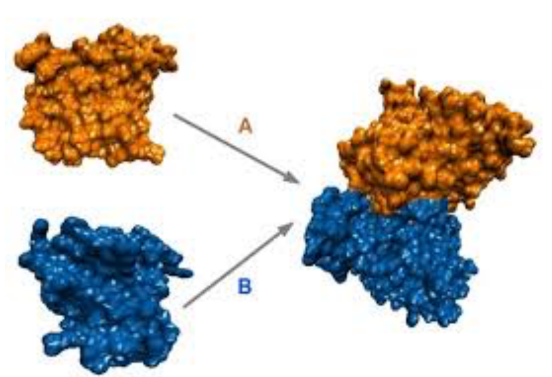
Thermodynamic and Biomolecules

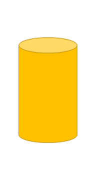
- Protein-protein interaction are a type of reaction.
- Meaning: thermodynamic parameters of the reactants will differ from the product.
- How can we calculate all of the thermodynamic parameters for protein-protein interactions?
- In the labs, we use ITC isothermal calorimetry techniques to calculate the parameters and also to study the affinity binding Ka and decoration Kd and also the stoichiometry (molar ratio).

9

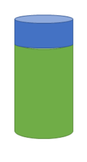
Protein-protein binding

- So, the biomolecules behave as same other non-biomolecules but they are in a closed system.
- Their parameters are changed during the reaction.

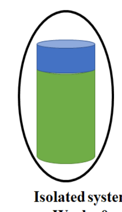




Open system



Close system



Isolated system
Work= 0

10

How does ITC work?

- Measurement principle of ITC
- We titrate a protein with another protein and then we measure the enthalpy ΔH of each point.
- By collecting different point, we can calculate the difference in (ΔH)
- When binding occurs, heat is either observed or released (comparing to the reference cell).
- As first injection is made, the micro-calorimeter measures all the heat releases till binding reaction has reached equilibrium.

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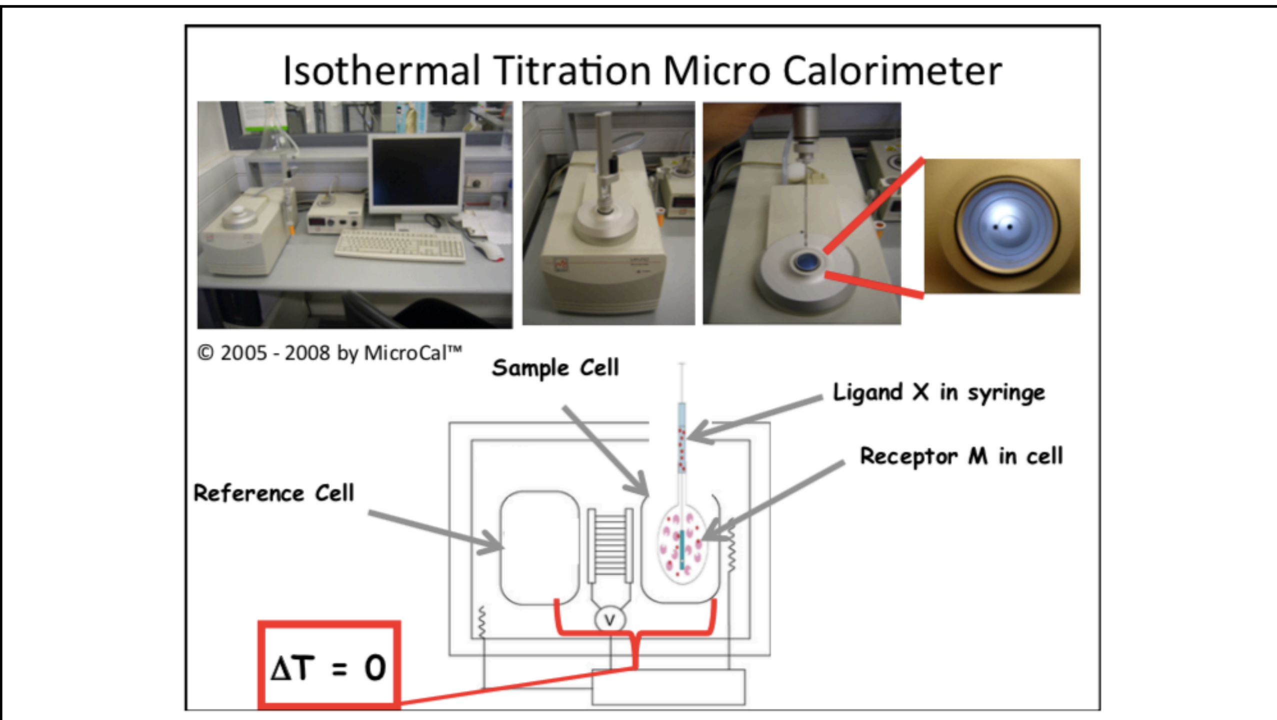

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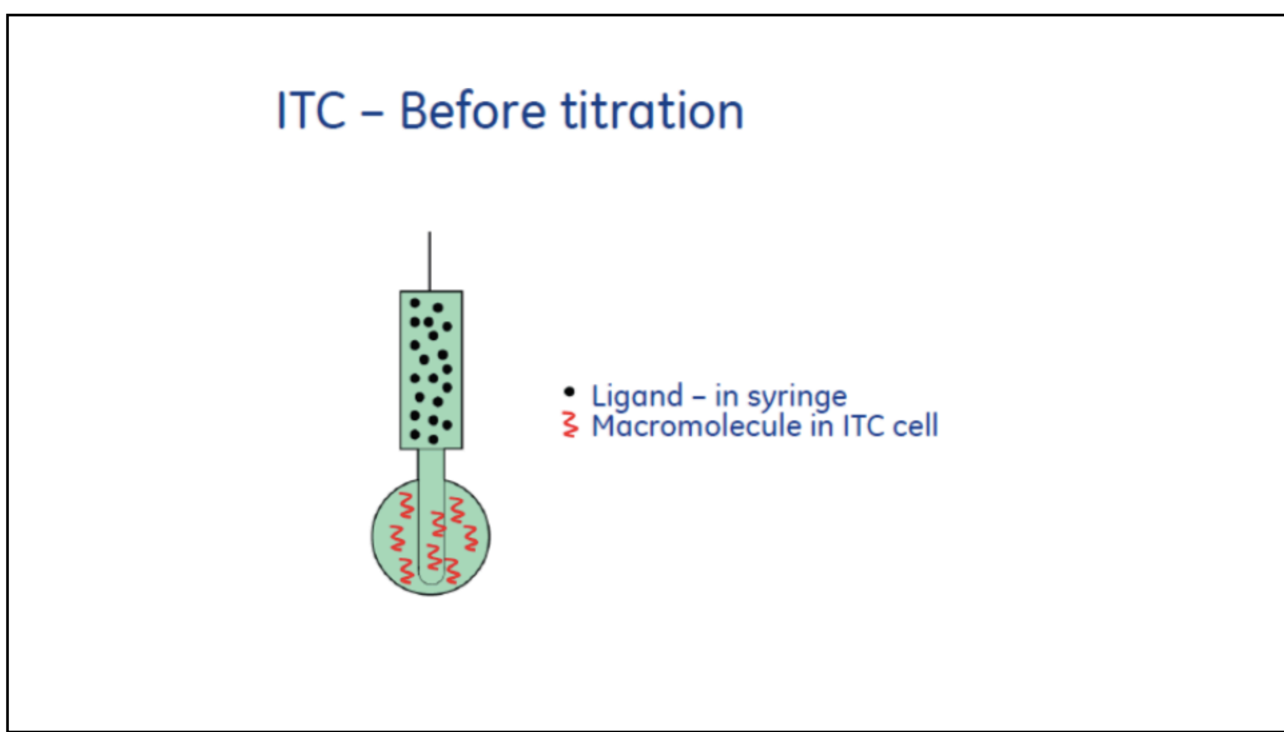

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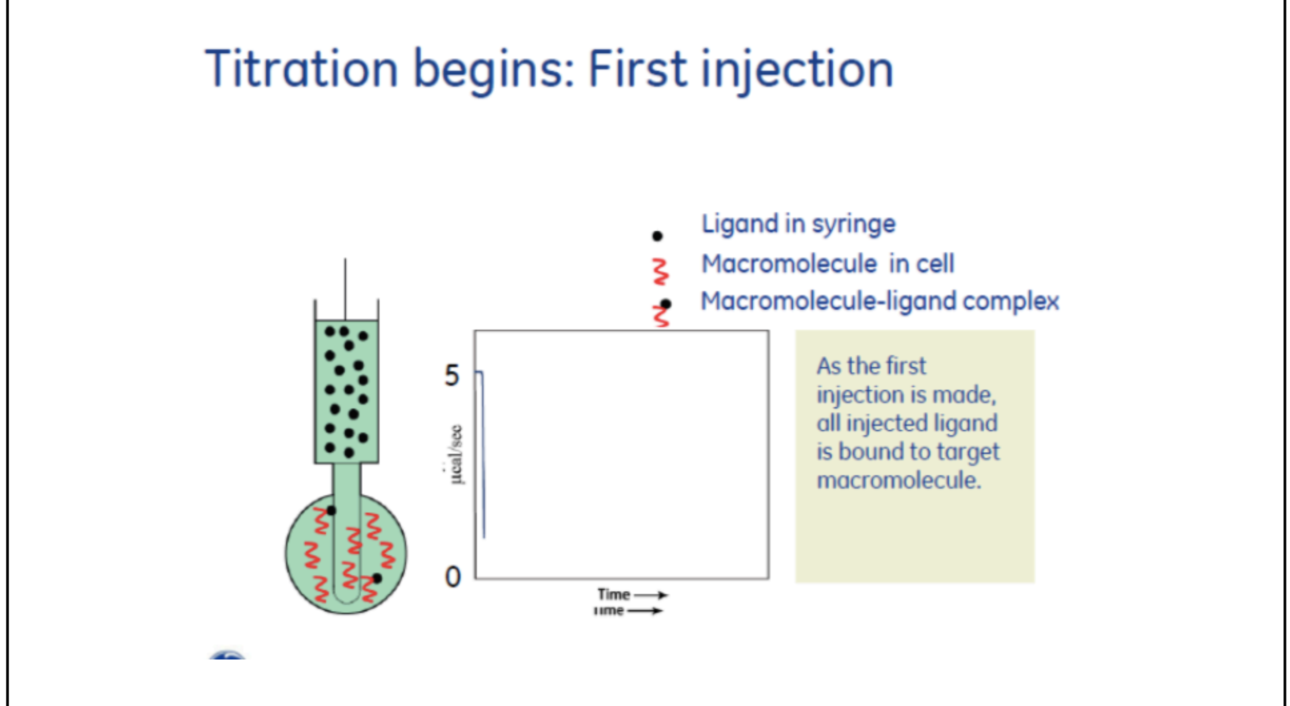

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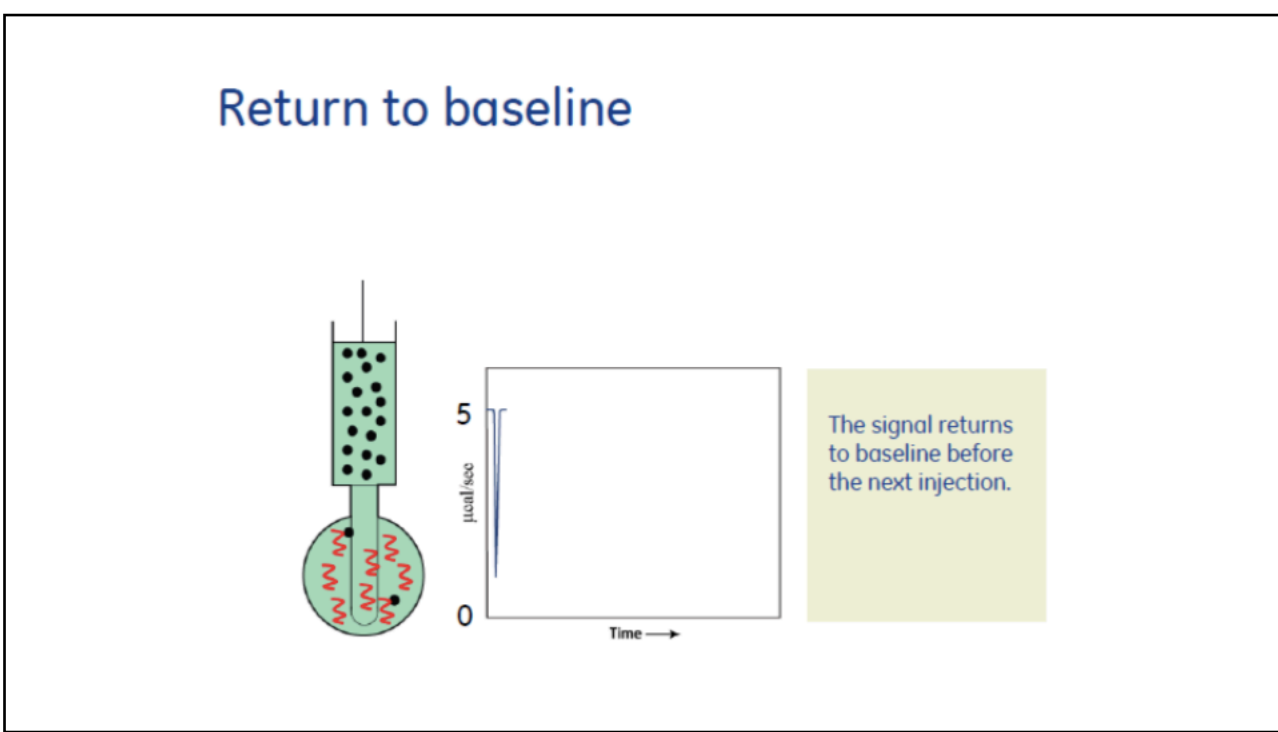

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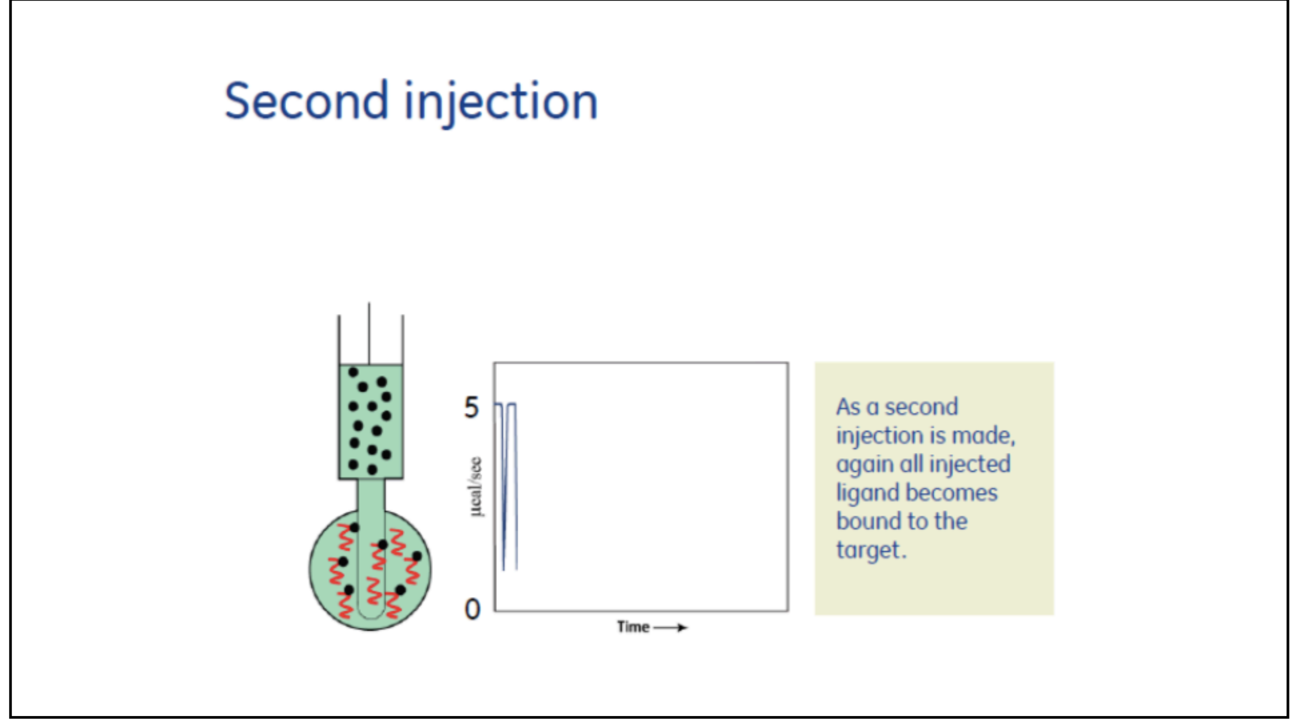

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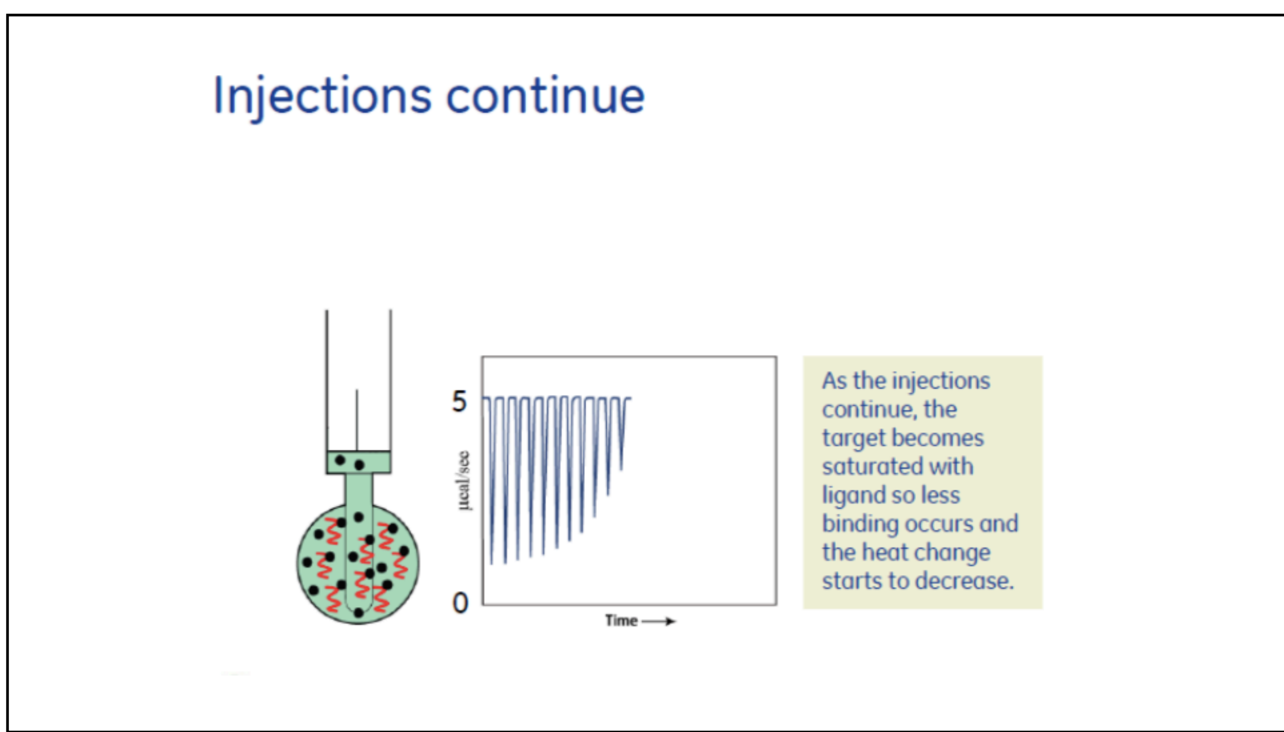

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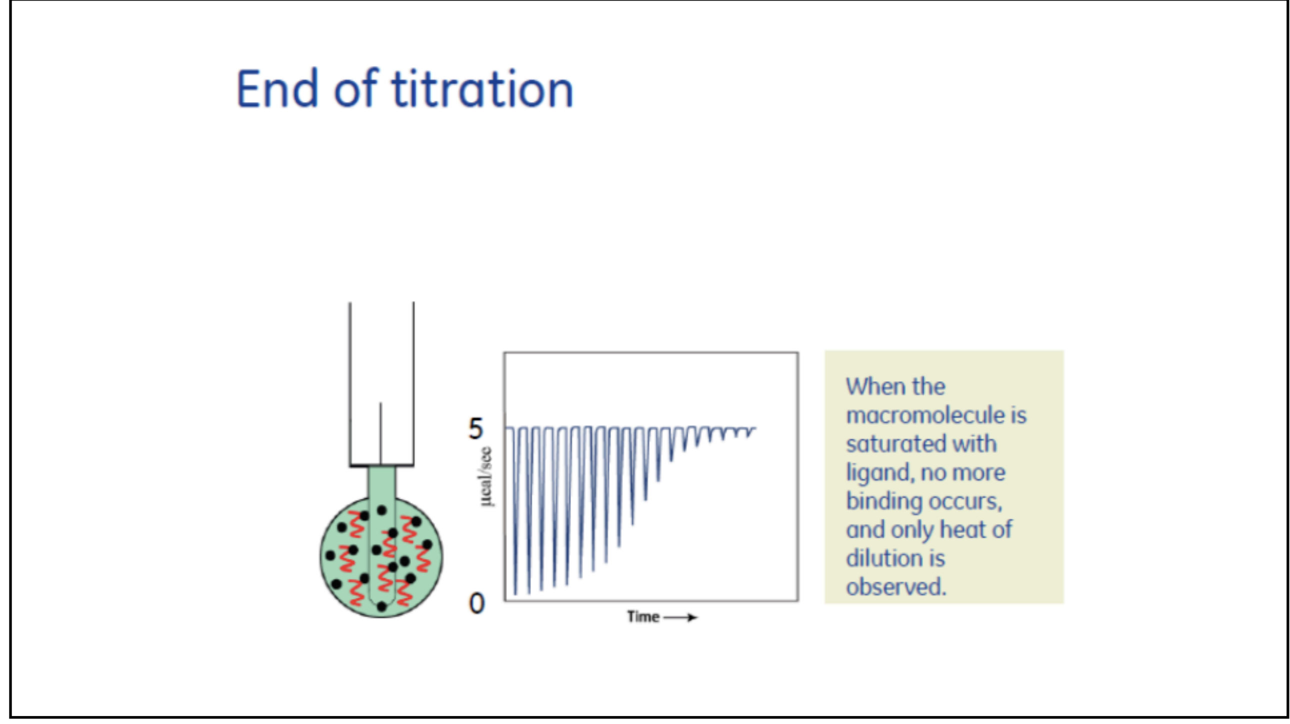

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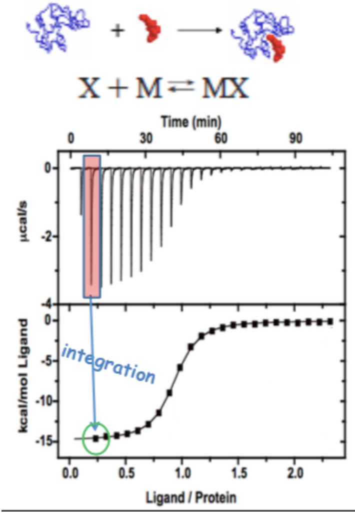
How do we fit the point to calculate the parameters ?

- We fit the collected points of ΔH to a curve equation to calculate the ka (affinity or association constant).
- Kd (dissociation constant)= 1/ka.
- When the binding is weak? Ka and kd
- When the binding is strong? Ka and kd



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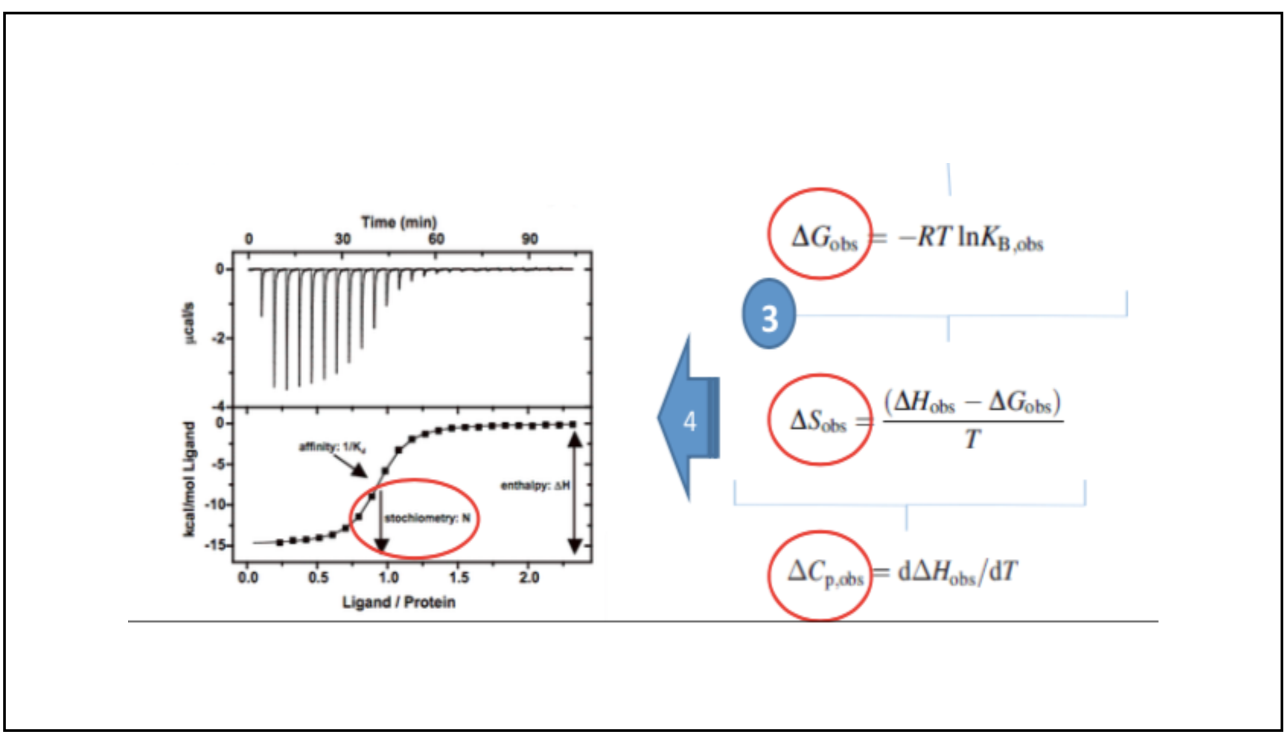
Other paramters?

- We can calculate ΔG form:
- ΔG = –RTlnΔKa = RTlnΔKd (Gibbs free energy equation), where: (R: gas constant and T is the absolute temperature).
- Or form ΔG = ΔH – TΔS (Van't Hoff equation).
- What does each tell?
- If ΔG= - or +
- If ΔH= - or +
- If ΔS= - or +
- What is ΔCp:

20



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Example of determining of thermodynamic parameters

Table 4.9 Determination of Kd of tropomyosin-troponin binding using ITC technique.

TPM	Stoichiometry n.	Kd (μM)	ΔS (cal/mol)	ΔH (kcal/mol)	ΔG (kcal/mol)
TPM1WTAS	1.66 ± 0.033	0.93 ± 0.47	5	-6.56 ± 2.6	-8.22
TPM1WTGA	1.27 ± 0.022	0.62 ± 0.04	3.9	-7.2 ± 0.2	-8.45
K6K7E	0.16 ± 0.15	4.9 ± 1.23*	-1500	-4546 ± 450	-7.23
K48K49E	2.04 ± 0.04	1.58 ± 0.03	11.8	-4.4 ± 0.2	-7.90
R90R91E	0.67 ± 0.01	0.25 ± 0.21	0.68	-8.7 ± 0.2	-8.98
S132R133E	1.48 ± 0.02	0.86 ± 0.43	13	-4.11 ± 0.11	-8.35
R167K168E	0.66 ± 0.02	0.79 ± 0.62	-39.1	-19.9 ± 0.8	-8.34
N202N203	0.68 ± 0.01	0.76 ± 0.90	-13.4	-12.3 ± 0.43	-7.61
R244S245E	0.87 ± 0.01	2.60 ±1.55	-8.77	-10.2 ± 0.9	-7.23

Note: the ΔG is shown in (kcal/mol) unit as it was calculated using the gas constants value (1.94 kcal $K^{-1}mol^{-1}$). For each parameter * indicates significant difference from TPM1WTAS ($p<0.025$). ANOVA oneway Post-Hoc method was used to analysis the data.

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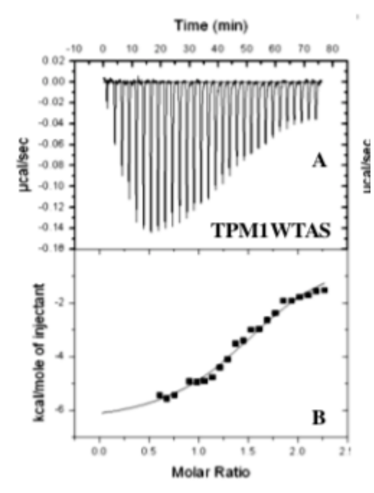
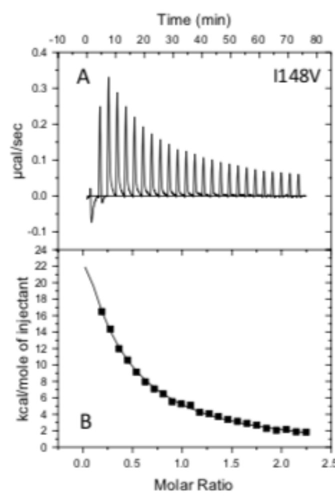
Endothermic and exothermic ITC

- Which one is Endothermic or exothermic reaction?





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What do we get from ITC/ why?

- The thermodynamic parameters tells:
- How much heat the biological system produces.
- Order or disorder? And how order the system is?
- How much energy does the biological system gives or take?
- How many biomolecules per each could bind? Stoichiometry.
- What is the affinity of the reaction? Too speed? Too tight? Or low affinity binding?

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Lecture 13

Mass spectroscopy for proteins

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1

What does mass spectroscopy check?

- Mass spectroscopy studies the proteomics.
- What are the Applications of the proteomics?

1. Protein identification – Specifically complex protein mixtures.
2. Study PTM post translational modification of the protein such as phosphorylation, acetylation a methylation etc.
3. protein molecular weight determination.
4. Structural studies by H/D exchange.

2

What does proteomics study?

- introduction to proteomics
- protein separation techniques
- proteolytic digestion
- peptide mass fingerprinting
- LC-MS/MS

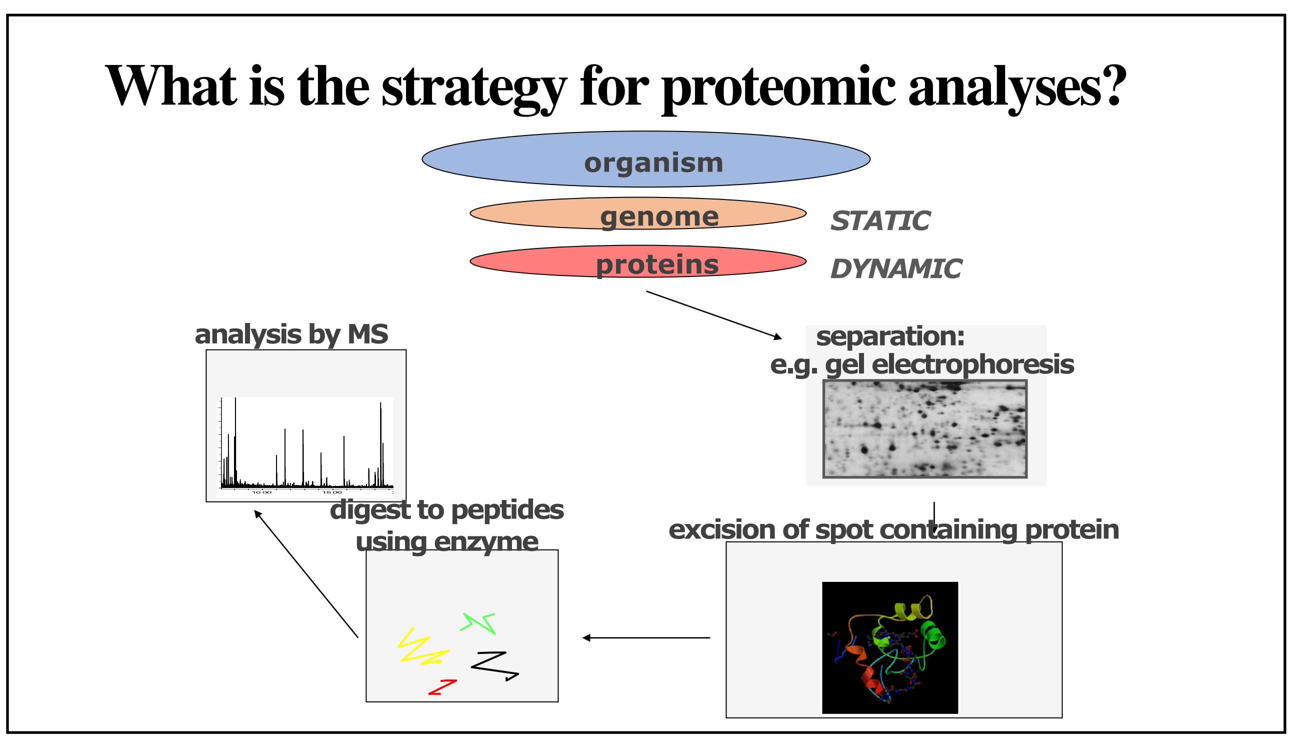
3

What is proteomics?

- The Identification AND Quantification of the full protein complement expressed by the genome of an organism at a particular point in time.
- It studies:

1. It finds which protein is present in the sample.
2. How much proteins are present in the sample.
3. Which gene the protein is belong to?

4



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separation techniques

multidimensional HPLC for separating Large numbers of proteins

cell lysate

↓

digest with protease

↓

load peptides onto SCX column
elute with increasing Salt concentration

↓

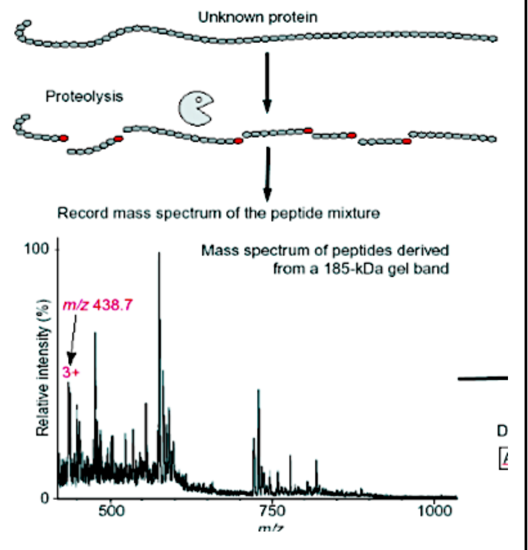
reversed-phase LC-MS

6

What is proteolytic digestion?

- It is a method to digest the protein by enzymes or chemical to make small fragments.

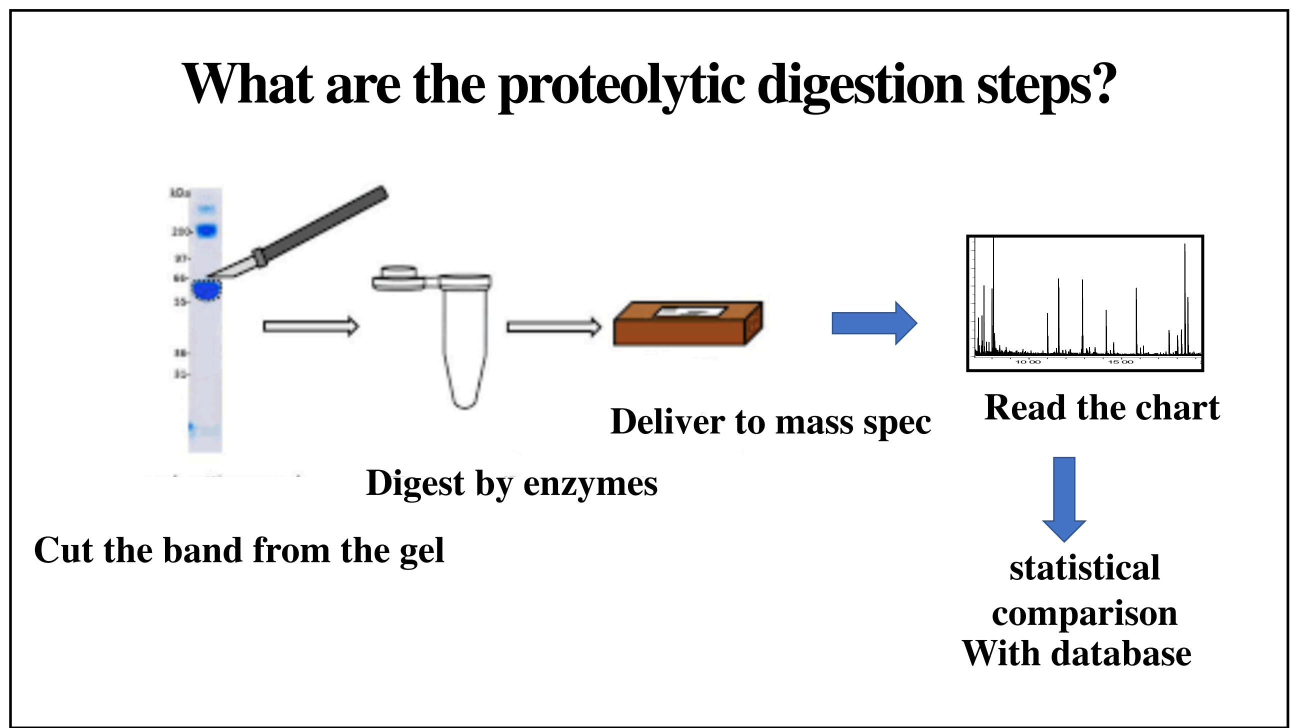
	cleavage sites	exceptions
Trypsin	C-term of **K** or **R**	if **P** is C-term of **K** or **R**
Chymotrypsin	C-term of **F**, **Y**, **W**, (M), (L)	if **P** is C-term of **F, Y, W**
CNBr	**C-term of M**	
Lys-C	C-term of **K**	
Arg-C	C-term of **R**	if **P** is C-term of **R**
Asp-N	N-term of **D**	
Glu-C (bicarbonate)	C-term of **E**	if **P** or **E** is C-term of **E**
Glu-C (phosphate)	C-term of **D** or **E**	
Pepsin (pH 1.3)	C-term of **F**, **L**	
Pepsin (pH >2)	C-term of **F**, **L**, **W**, **Y**, **A**, **E**, **Q**	
Proteinase K	C-term of **F**, **L**, **W**, **Y**, **A**, **V**, **I**	



Mass spectrometric identification of an unknown protein can be obtained from determining the sequence of proteolytic fragments and comparing the sequence with available databases

7

What are the proteolytic digestion steps?



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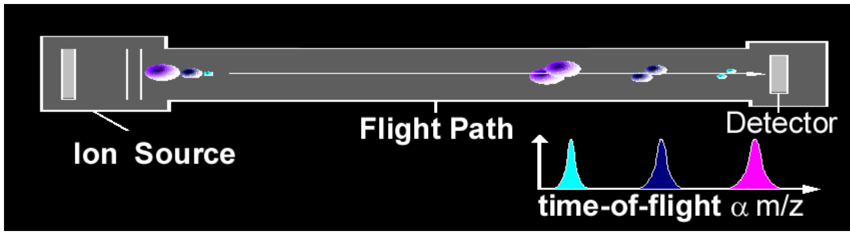
How does mass work?

Mass determination by Time-of-Flight



1. ions generated in source
2. accelerated using high voltage
3. drift to detector
4. time-of-flight inversely proportional to m/z

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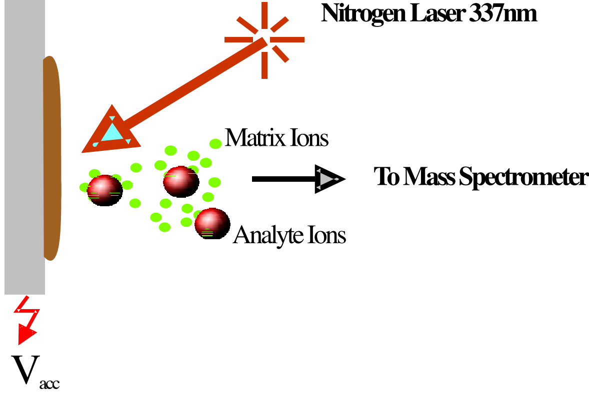
What is used to ionize the molecules?

Laser Desorption/ionisation



Charge transfer occurs between Matrix Ions and Analyte molecules

$M + H^+ \longrightarrow [M+H]^+$

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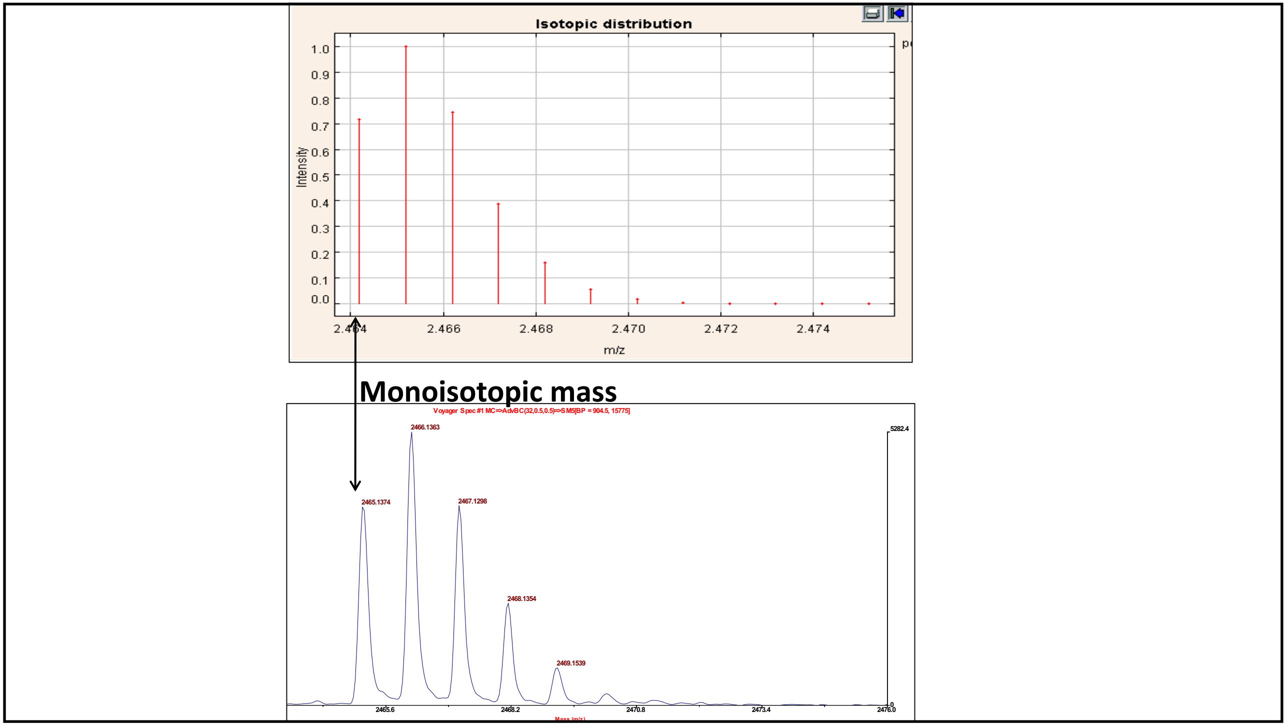

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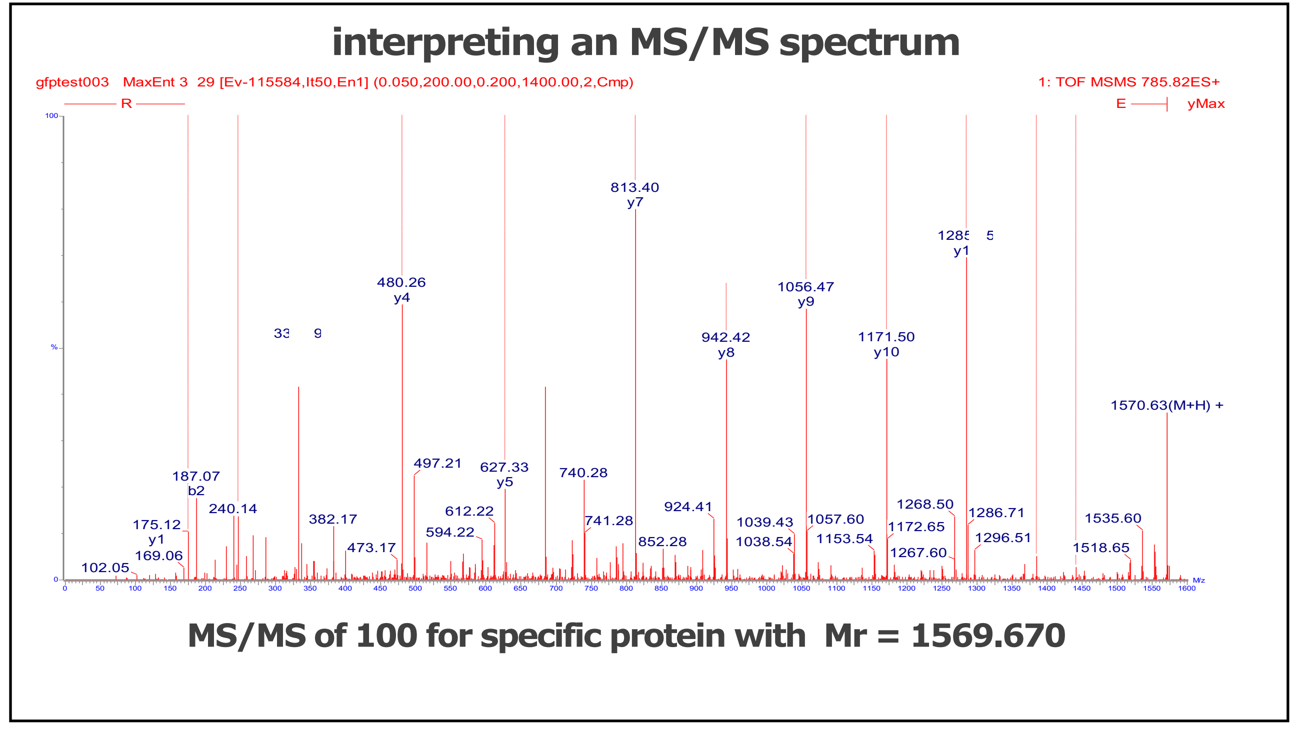

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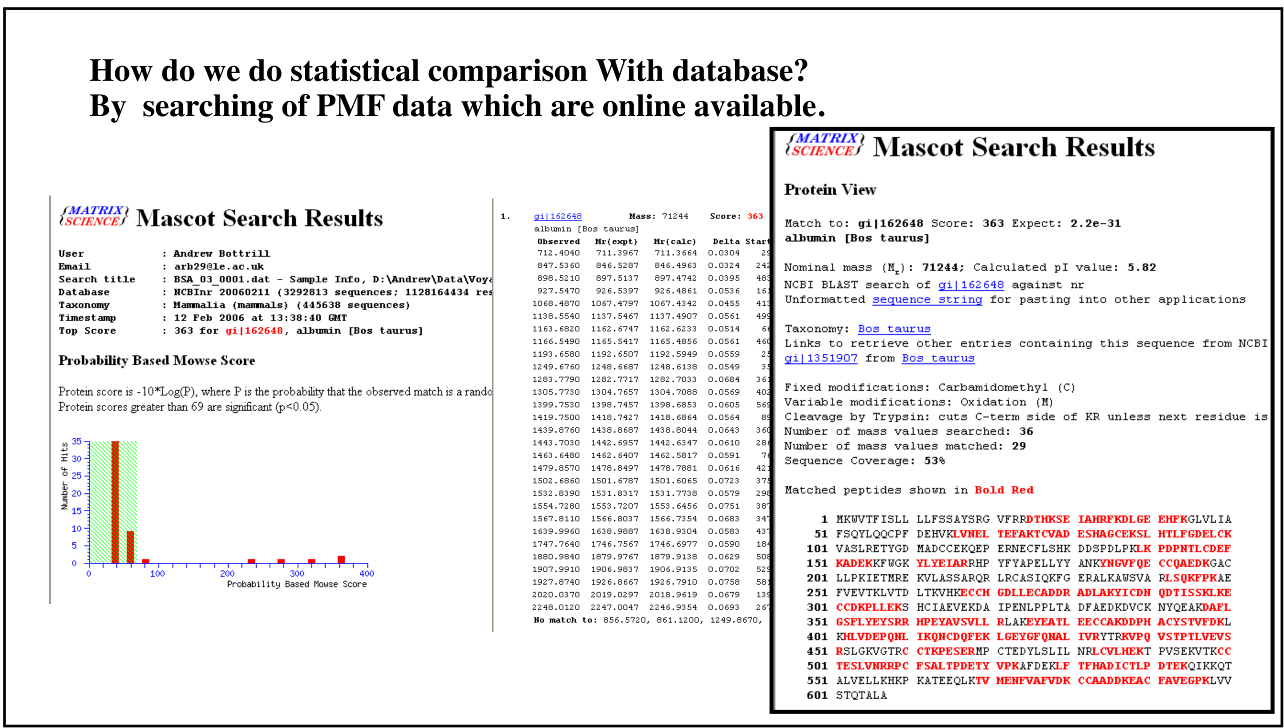

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