Protein separation and characterisation

Separation of protein mixtures

- Relies on differences in physico-chemical properties
- Charge, size, ligand affinity, solubility, hydrophobicity, thermal stability

Column chromatography

- A protein sample applied to column equilibrates between the stationary and mobile phases
- Proteins with certain characteristics will bind to the stationary phase
    - Those lacking the characteristic remain in the mobile phase and pass through the column
- Final step involves displacing the protein from the stationary phase – "elution"
    - Introduce a particle that competes with the protein binding site on the stationary phase
- Separation by charge – ion exchange chromatography
    - Protein mixture added to column containing cation exchangers
    - Negatively charged stationary phase – more negative proteins move faster, elute earlier
- Size-exclusion chromatography or gel filtration
    - Protein mixture added to column containing cross-linked polymer
    - Proteins separate by size – larger molecules pass more freely, elute earlier
- Separation by affinity chromatography
    - Mixture added to column containing polymer-bound ligand specific for protein of interest
    - Binding affinity exploited – protein of interest is eluted by ligand solution

Electrophoresis for protein analysis

- Electric field pulls proteins according to their charge – separation
- Gel matrix hinders mobility of proteins according to their size and shape
- SDS-PAGE – separate proteins according to size only – small move faster
    - SDS (a detergent) binds to, and unfolds all the proteins – gives all proteins a uniformly negative charge; native shape of proteins does not matter

Edman protein sequencing

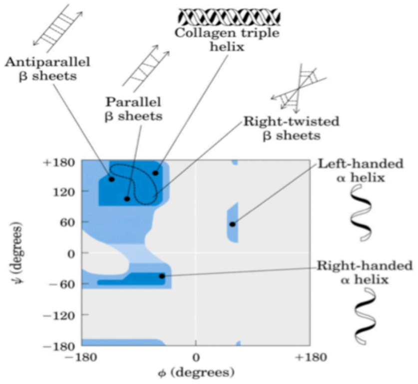
- Edman degradation labels and removes the terminal residue from a peptide (by hydrolysing the terminal peptide bond)
    - Residue is a derivative of the amino acid
- Repeating the procedure provides the amino acid sequence

Spectroscopic detection of aromatic AAs

- Aromatic AAs absorb UV light maximally at 280nm
- Concentration determined using Beer's law $A = \varepsilon cl$
- Tryptophan absorbs 4x as much as tyrosine (at 280nm)

Ramachandran plots

- For secondary protein structure
- Darker colour = more amino acids
- Good indicator of the quality of a structure
- Most proteins occur where ϕ and ψ are 60^{o} and -60^{o}
    - This is where they are most energetically stable



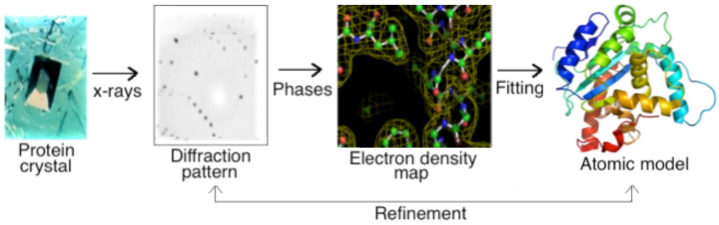
X-ray crystallography	**NMR**
Solid state (static)	Solution phase (dynamic)
Disordered regions problematic	Disorder built in – structure assumed to 'breathe'
Quality crystals needed	Pure protein in any form can be used
Cannot see hydrogens (too small for x-rays to hit)	Sees only protons (H^+)
Produces a single structure	Produces a series of structures (due to movement)
High power x-ray source needed	High field NMR needed
More detailed	Less detailed structure produced

Protein structure determination

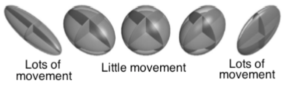
X-ray crystallography

- Use an x-ray defractometer
- Crystal – made of multiple 'units', each containing the same molecule
  - Increases likelihood of detection, raises signal to measureable level

1. X-rays hit rotating crystal, atom's electrons scatter the x-rays – produces diffraction pattern
2. Turn diffraction pattern into electron density map using Fourier transformation
   - Requires both amplitude *and* phase of diffracted waves – need to deduce phase indirectly
   - EDM – interpret diffraction pattern as a plot of electron density vs. spatial arrangement
   - Diffraction is caused by electron clouds
   - Higher atomic number = greater density = larger electron clouds = greater scattering
3. Model building – EDP is interpreted as a set of atomic coordinates
   - Fit a protein backbone, then insert the amino acid sequence
4. Refinement – improve phases, to produce clearer maps and better models



Overcome phase problem	Explanation
Multiple Isomorphous Replacement (MIR)	- Heavy atom method - Multiple crystals of the same protein, but with different heavy metals – compare relative scattering
Multiple wavelength Anomalous Diffraction (MAD)	- Anomalous scattering - Near absorption wavelengths, metals displays unequal Friedel pairs
Molecular Replacement (MR)	- Similar protein already solved - Use fragments of known to estimate phases of unknown structure
Brute force "shake 'n' bake"	- Multiple guesses at solution – very computationally expensive



- X-ray crystallography may also produce "temperature factor" for each atom
  - Degree of movement depends on temperature
  - Affects choice of drug site (less movement = easier insertion)
- Smaller resolution = greater accuracy = greater detail (ideal: <2.05Å resolution)
- The lower the Crystallographic R-Factor value, the better the structure is (given as % or 0.X)

Nuclear Magnetic Resonance (NMR) Spectroscopy

- Information about nuclei conformation, where atoms are located in the molecule, and dynamics
  - Insert drug where drug is more static, than areas where movement is high
- A nucleus with an odd atomic or mass number has a nuclear spin that generates a magnetic field
  - When placed in an external magnetic field, spinning protons act like bar magnets
  - Magnetic fields of spinning nuclei will align either *with* or *against* the external field
  - A photon with right amount of energy can be absorbed, causing the spinning proton to flip
  - Magnetic field strength must be increased for a shielded proton to flip at the same frequency as a naked proton
- Produces signals on a spectrum
  - Number of signals – how many different kinds of protons present
  - Location of signals – downfield (left) = proton is less shielded; upfield = more shielded
  - Intensity of signals – number of protons of that type
  - Signal splitting – number of protons on adjacent atoms
    - n = no. hydrogen atoms attached to the adjacent carbon
    - n+1 = how many peaks will be seen in the cluster
- TOCSY and NOESY – determine relative distances between each proton to obtain structure