

TD5

Xray, NMR, and Cryo-EM methods for determining the structures of macromolecules

All three methods very important.

Evaluate them against one another in terms of **spatial resolution** (what structures can you see? Single C-C-bonds? Or overall protein shape only?), **temporal resolution** (one snapshot only? Or dynamic information as the protein is changing over time?), **preservation of native conditions** (does method analyze soluble protein in water at 37 C? or does the sample need to be frozen or crystallized?), and **sample requirements** (purity, amounts, complexity).

X-ray Crystallography

- Summary: take extremely pure and homogeneous sample and crystallize it. Irradiate crystal with xray beam. A detector records the diffraction pattern and a computer reconstructs the atomic structure of the molecule.
- Atomic resolution - can see individual atoms and bonds, down to $\sim 1 \text{ \AA}$. This is because the structure is “visualized” with x-rays, which have a wavelength similar to the length of a bond (unlike visible light, whose wavelength is $\sim 5000 \text{ \AA}$)
- No temporal information: static snapshot of protein, no kinetic intermediates, etc
- Protocol:
 - o Make extremely pure sample. Use black magic to crystallize it (common technique is the hanging drop method).
 - o Cool crystals rapidly to near-liquid nitrogen temp to reduce thermal vibrations (increases resolution)
 - o Mount crystal on a pin in the path of xray beam
 - o Irradiate with xray beam; a detector records the diffraction pattern
 - o Rotate the crystal, collect more diffraction patterns
 - o Repeat with an identical crystal soaked in heavy metal ions
 - o A computer algorithm converts these diffraction patterns into 2D electron density maps, showing where atoms are located (note: since hydrogen has only one electron, it is difficult to map hydrogens). The heavy metal crystal is for helping with “phasing”, or figuring out the phases of the waves that formed each spot on the diffraction pattern – essential for getting an electron density map.
 - o Combining many 2D electron density maps gives a 3D map
 - o Build a model of the protein to fit the map, given knowledge of the amino acid sequence and knowledge of what is chemically reasonable (“model building” and “model refining” process)
 - o R factor: the gold standard
 - 0.59 = random (0.35-0.55 = typical starting model before refinement)
 - >0.25 = mistakes in overall fold possible
 - >0.2 = good, maybe some sidechain errors
 - <0.2 = excellent, no major problems

- Rfree = R calculated from a random subset (5%) of data before refinement; should be <0.3 and within 0.1 of R.
- Advantages:
 - Best resolution
 - No size limitation for protein under study (in contrast to NMR)
- Disadvantages:
 - Technically very challenging to make crystals of proteins. Heterogeneous samples, membrane proteins, complexes of proteins – all very challenging
 - Static snapshot

NMR (Nuclear Magnetic Resonance)

- Summary: An aqueous protein sample labeled with ^{13}C and/or ^{15}N is placed in a large magnet. An external magnetic field is applied and the, through-bond (J coupling) and through-space (NOE) coupling constants are measured. These provide a set of distance constraints between pairs of ^{13}C or ^{15}N nuclei, leading to a structural model of the protein.
- NMR detects chemical shifts of atomic nuclei with nonzero spin (^1H , ^{13}C , ^{15}N). The shifts depend on the electronic environments of the nuclei, namely, the identities and distances of nearby atoms.
- Structural information comes from measurements of through-bond (scalar or J coupling) or through space (the nuclear Overhauser effect NOE) magnetization transfer between pairs of protons. J couplings between pairs of protons separated by three or fewer covalent bonds can be measured. The intensity of a NOE is proportional to the inverse of the sixth power of the distance separating the two protons and is usually observed if two protons are separated by $< 5 \text{ \AA}$.
- The result of an NMR experiment is an ensemble of protein structural models, rather than a single structure.
- Protocol:
 - Label protein with ^{13}C and/or ^{15}N
 - Make concentrated protein solution in water (0.2-1 mM, 6-30 mg/mL)
 - Apply external magnetic field to sample; ^{13}C and ^{15}N nuclei will undergo precession (spinning like a cone), with a frequency that depends on the external environment
 - From these frequencies, computer determines the J coupling constant and NOE value between every pair of NMR-active nuclei
 - These values provide a set of estimates of distances between specific pairs of atoms, called "constraints"
 - Build a model for the structure that is consistent with the set of constraints
- Advantages:
 - Pretty good resolution 2-5 \AA , better than cryo-EM, worse than x-ray
 - Native like conditions – sample is hydrated, not frozen, and not in a crystal lattice

- Can get dynamic information – observe conformational changes etc
- Can look at relative disorder of specific regions of a protein – can see if a loop is static or flexible over time
- Disadvantages:
 - Not as high resolution as x-ray
 - Require a lot of protein to get a good signal
 - Require very concentrated samples (can get insoluble aggregates)
 - Structure of mixed species cannot be determined
 - Limit on protein size measurable, since molecule must tumble rapidly to give sharp peaks. Typically, proteins must be <30kD. Best case 100 kD.

Cryo-EM (Cryo-Electron Microscopy)

- Summary: an aqueous biological sample is frozen rapidly and irradiated with a beam of electrons. A detector senses how the electrons are scattered and a computer reconstructs the 3D-shape of the molecule.
- Review: Method: Acta Crysta 2000, D56, 1215. Application to ribosome: BioEssays 2001, 23, 725.
- Spatial resolution: Best ~8 Å, in practice >10-15 Å for single particle EM
- Temporal resolution: more or less static snapshot, but some people have examined kinetic intermediates by mixing components and then flash-freezing in 5 msec
- Tradition EM: Samples were stained with dye and dehydrated. Leads to sample distortion so data doesn't reflect native structure of molecule
- Ribosome structure observed at 8-11 Å – good enough to see major/minor grooves of RNA helices, and protein domain structures. (First ribosome structure 1991 at 45 Å)
- Cryo-EM has also been used to look at conformational changes during tRNA binding and protein factor docking. Address such questions as: what is the path of the mRNA and polypeptide chain? Where do tRNA and factors bind? How does ribosome change conformation to promote these events?
- Protocol:
 - Dilute the sample so that only a few macromolecules will be present in the field of view (for “single particle EM”)
 - Put a droplet of the sample onto a carbon support film with holes in it
 - Blot away droplet with tissue, leaving just a thin film of liquid
 - Flash freeze sample in liquid ethane to embed sample in a thin layer of vitreous (non-crystalline, glass-like) ice (thickness <2000 Å)
 - Place sample in cooled EM instrument
 - Irradiate with focused, coherent electron beam (electron wavelength ~0.025 Å)
 - Collect projected 2D image (generated from scattered electrons)
 - Repeat thousands of times for thousands of individual molecules at various orientations (ex. for ribosome 73,000 times)
 - Computer reconstructs 3D structure of molecule

- Advantages:
 - Preserves the native structure of the sample (sample remains hydrated)
 - Need only tiny amount of sample (a few 1000 molecules)
 - Very large molecules/assemblies ok
 - Heterogeneous samples ok (protein complexes, etc)
 - Can get kinetic snapshots of various conformational states/intermediates
 - No sample treatment required (no isotopic labeling, no isomorphous replacement)
 - Low temperature reduces radiation damage to sample from electron beam

- Disadvantages:
 - Resolution not as high as NMR or xray
 - Single particle cryo-EM only works on samples >several hundred kDs
 - Expensive instrumentation
 - Sample prep technically difficult – need perfect ice thickness, no contamination, proper concentrations
 - Sample is frozen, not at room temp
 - Sample can be damaged by intense electron beam (C-H bond breakage, C-C bond formation)
 - Low signal to noise of images. Without a staining step, and with low intensity beam to minimize sample damage, resulting image is very noisy. Typically data from 1000s of images must be averaged using computer algorithms. This is why resolution is low.