JBC REVIEWS

Seeing the PDB

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Jane S. Richardson[1,*], **David C. Richardson**[1], **and David S. Goodsell**[2,3,*]

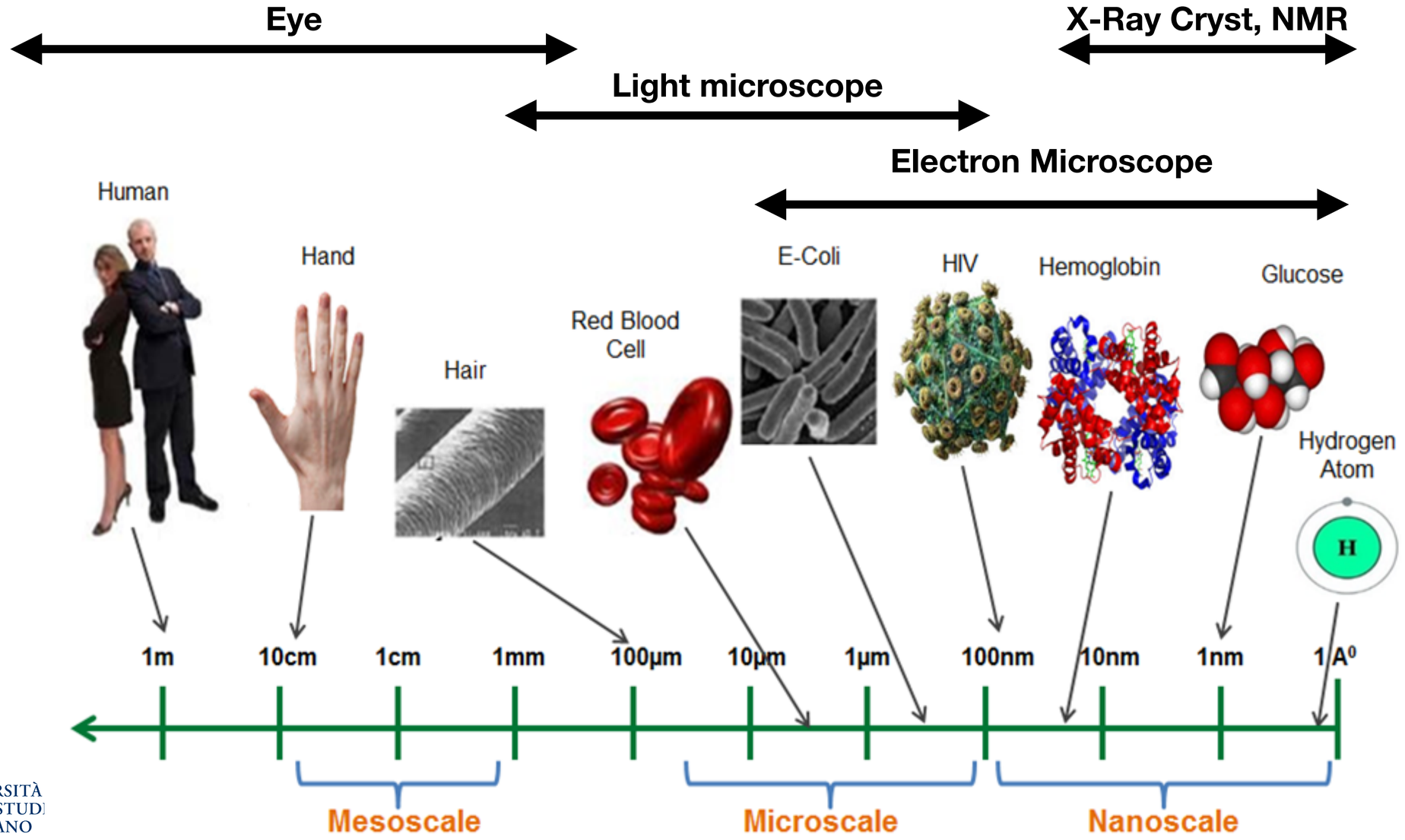
From the [1]Department of Biochemistry, Duke University, Durham, North Carolina, USA; [2]Department of Integrative and Computational Biology, The Scripps Research Institute, La Jolla, California, USA; [3]Research Collaboratory for Structural Bioinformatics Protein Data Bank, Rutgers, the State University of New Jersey, Piscataway, New Jersey, USA

Edited by Karin Musier-Forsyth

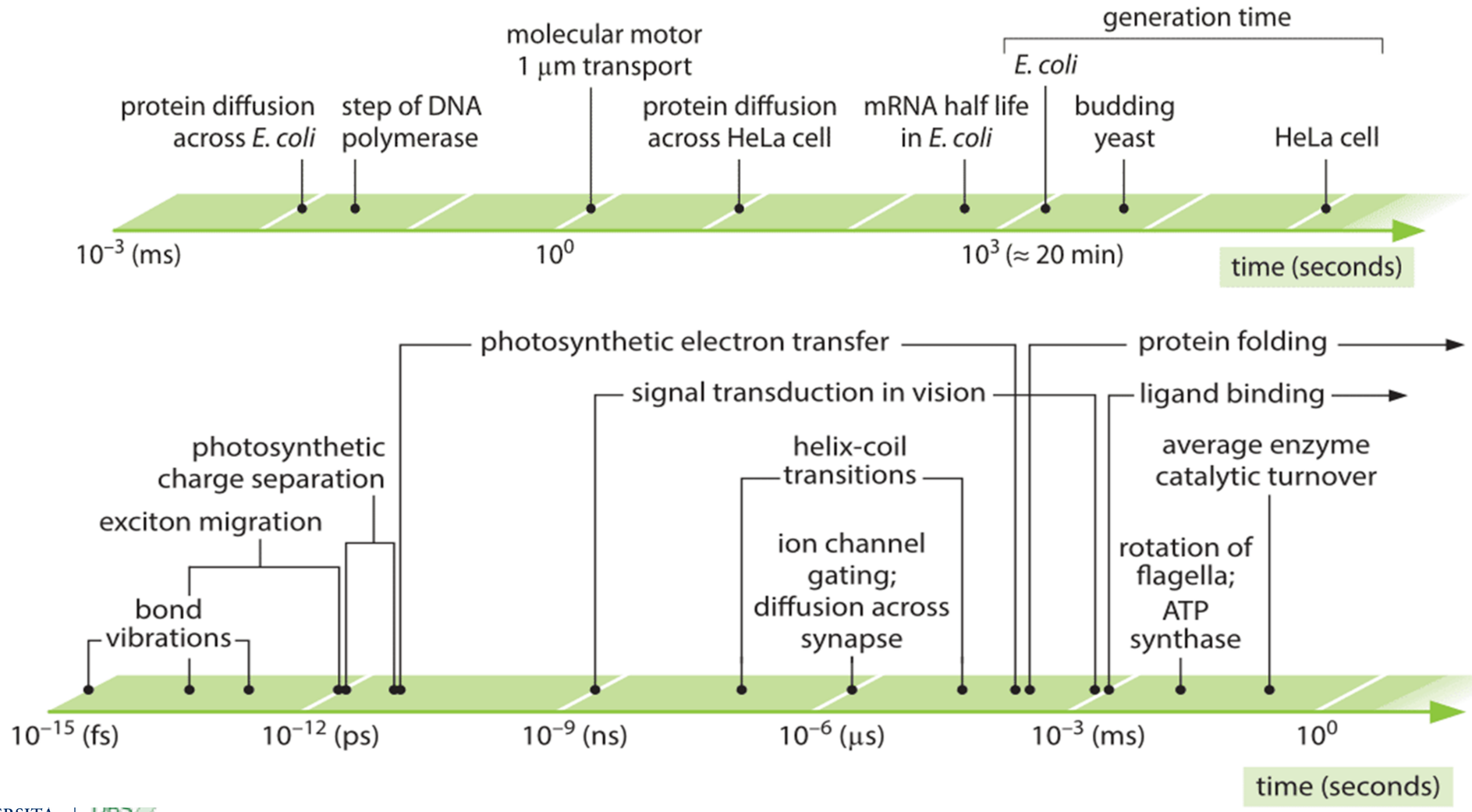
Biomolecules, Structural Biology and Visualisation

Structural Bioinformatics

Light microscope cannot look at molecular spatial scales



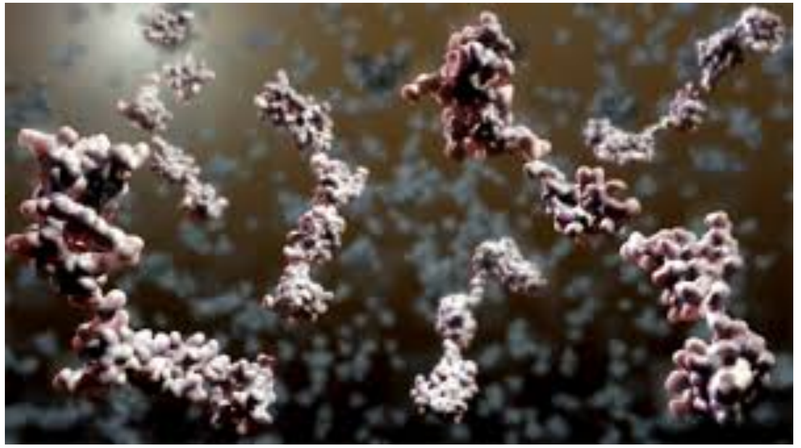
Molecules motion can span many time scales



As a consequence: observing the microscopic world is hard

We cannot use light microscopes, so we don’t see directly the molecules, we need to interpret other kind of signals (shorter wavelength, electrons, magnetic fields, …)

We do not look at a single molecule at a time but at many (~fraction of Avogadro number) with issues of rotations and translations



As a consequence: observing the microscopic world is hard

Molecules have different sizes, and move on multiple time scales

Long exposure: good signal vs noise ratio, but molecules that move fast may become messy

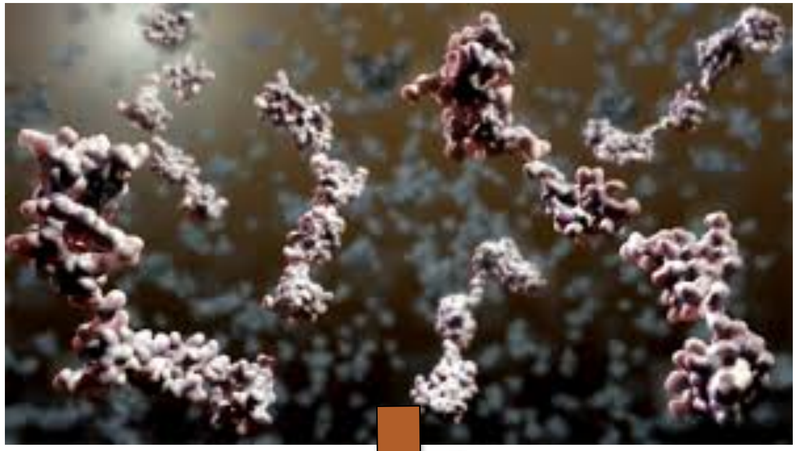
Short exposure: you could resolve the motion of all molecules, but no signal

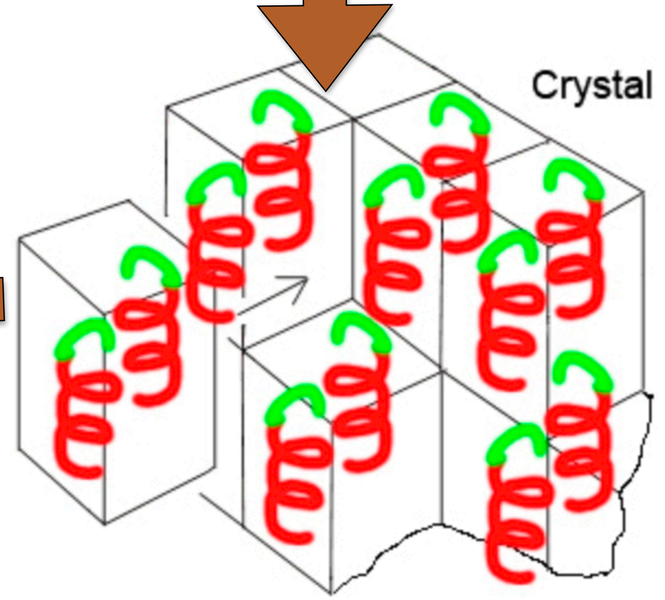
Finding an optimal compromise is not always possible

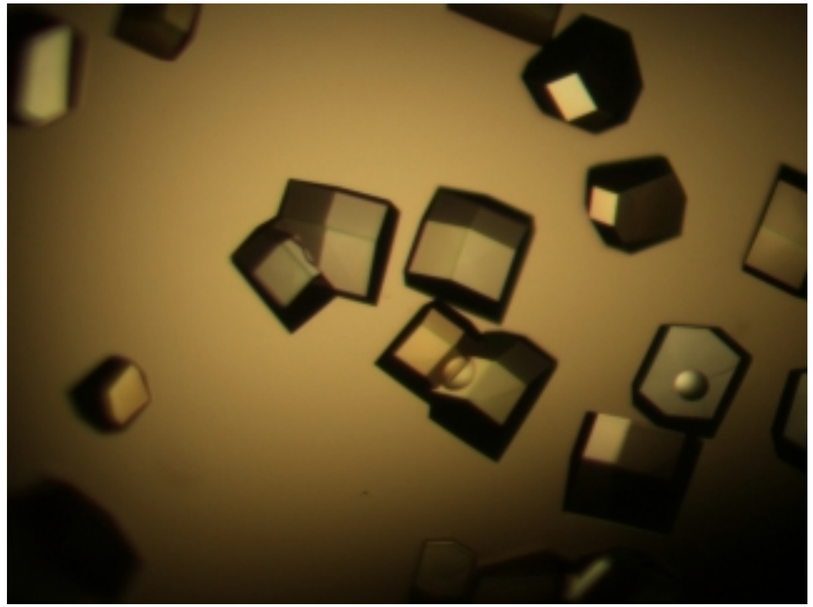
So how do we “take pictures” of molecules? 1. X-ray crystallography

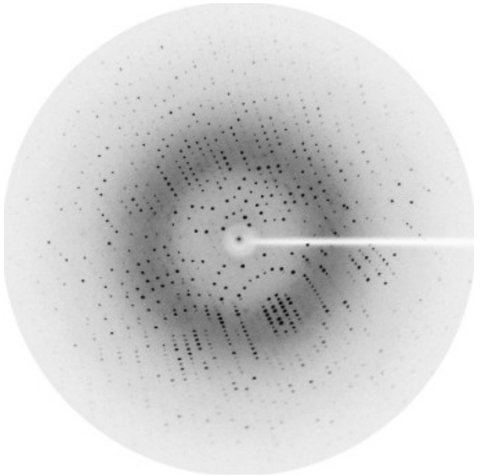
Issues are signal/noise, small size, motion on many time scales

SOLUTION: We make all molecules adopt the same structure in an ordered 3D organisation: a crystal, that we observe using X-rays





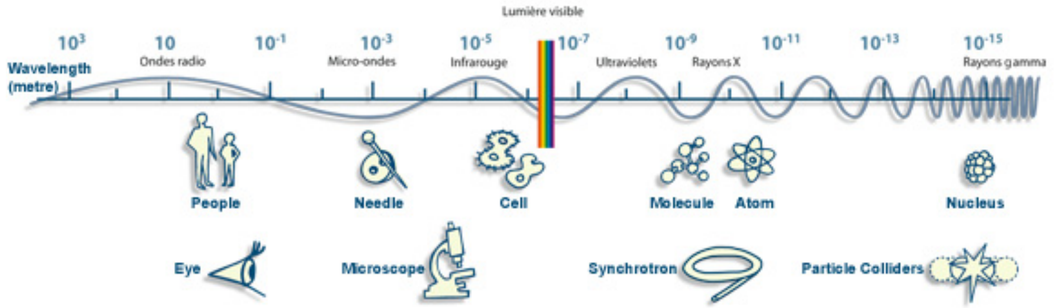




X-ray Crystallography

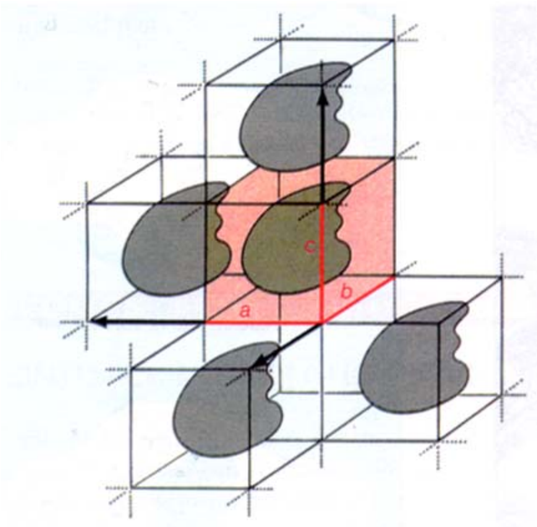
Why X-rays ?

The wavelength of a X-rays is roughly **1 Å** (10^{-10} m), which is on the scale of a single atom, and it allows to have **sufficient resolution to determine the atomic positions**

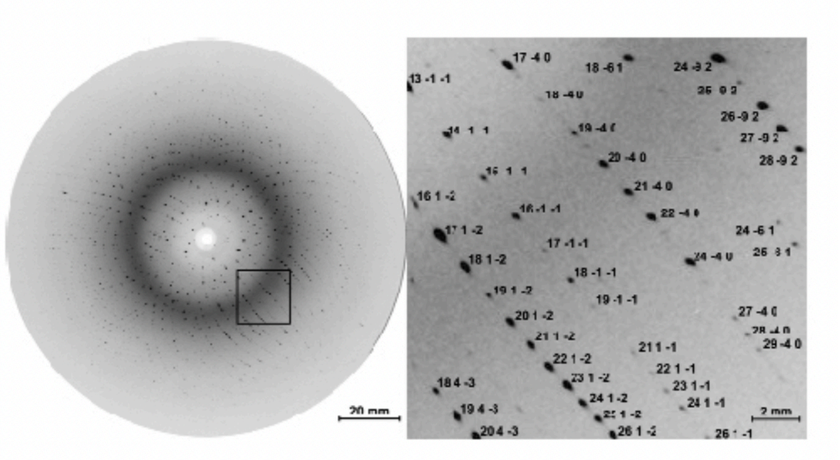


Why crystals ?

X-ray crystallography requires a crystal to amplify the signal (**10^{15}-10^{16} identical molecules**); the periodicity of the electron density is used to diffract the X-rays with manageable measurement error



X-ray Crystallography



The crystal is rotated and multiple images are collected to find out the symmetry of the crystal, that is to each black spot of each image you can assign 4 numbers: the intensity and three coordinates h, k, l (Miller indices): I_{hkl}

Each I_{hkl} is associated with a Structure Factor F_{hkl} that is associated with the electronic density $\rho(x,y,z)$ of the molecule in the crystal. With a computational procedure it is possible in some cases to obtain the electronic density that is an envelope in 3D that is then used to reconstruct the 3D coordinates of the atoms of the molecule.

$$I_{hkl} \propto I_0 \frac{V_{xtal}}{V_{Cell}^2} |F_{hkl}|^2$$

not measured

$$\rho(x,y,z) = \frac{1}{V} \sum_{hkl} |F_{hkl}| \exp(i\alpha_{hkl}) \exp[-2\pi i(hx+ky+lz)]$$

What does X-ray crystallography tell us about molecules motion?

The key idea of X-ray crystallography is that of removing protein motion. Both the motion of the different molecules, that are trapped in the crystal lattice, but also the internal motion. The structure that is obtain in crystal is generally accepted to be representative of the most populated structure found in solution.

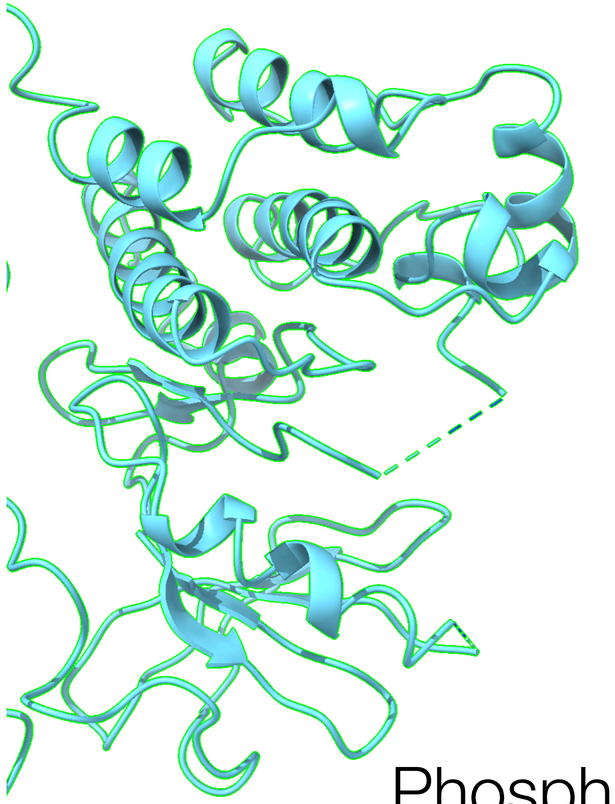
We will see later that solution techniques like NMR and SAXS can be used to test this assumption case by case. But what do the molecules in the crystal can tell us about their motion?

What does X-Ray crystallography tell us about molecules motion?

1. Molecules that are too flexible (intrinsically disordered proteins, proteins with many disordered loops, or with long tails) generally do not crystallise.
2. Even in proteins that do crystallise, some regions or even single atom, do not scatter and are then invisibile in the 3D density (there are regions with missing density), these regions are generally deemed to be either too flexible or to populate multiple conformations in the crystal
3. Some times the same atoms scatter in different ways allowing to find multiple conformations for the same atoms in the crystal (crystal anisotropy), these motions are generally interpreted to exists also in solution

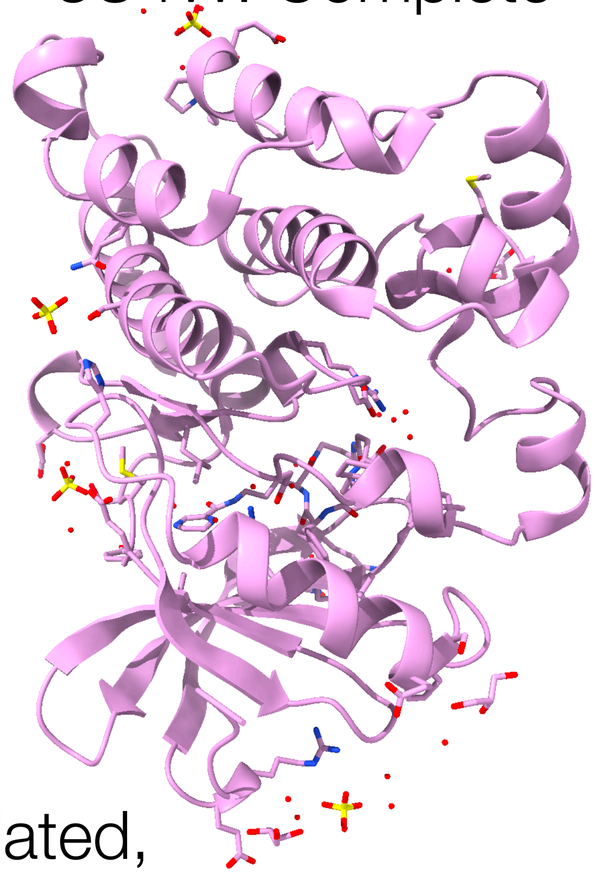
Example: the active-loop of C-src Kinases

2PTK: Missing 407-424



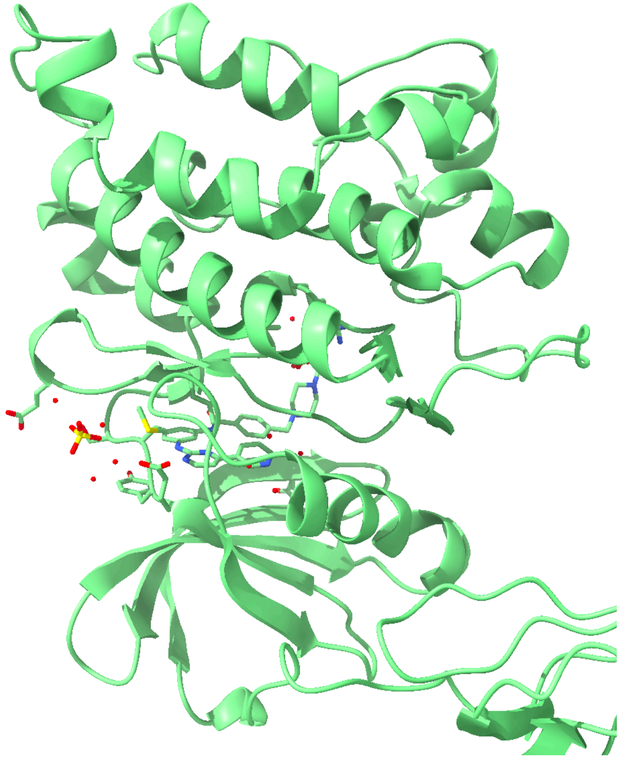
Phosphorylated, inactive state

3U4W: Complete



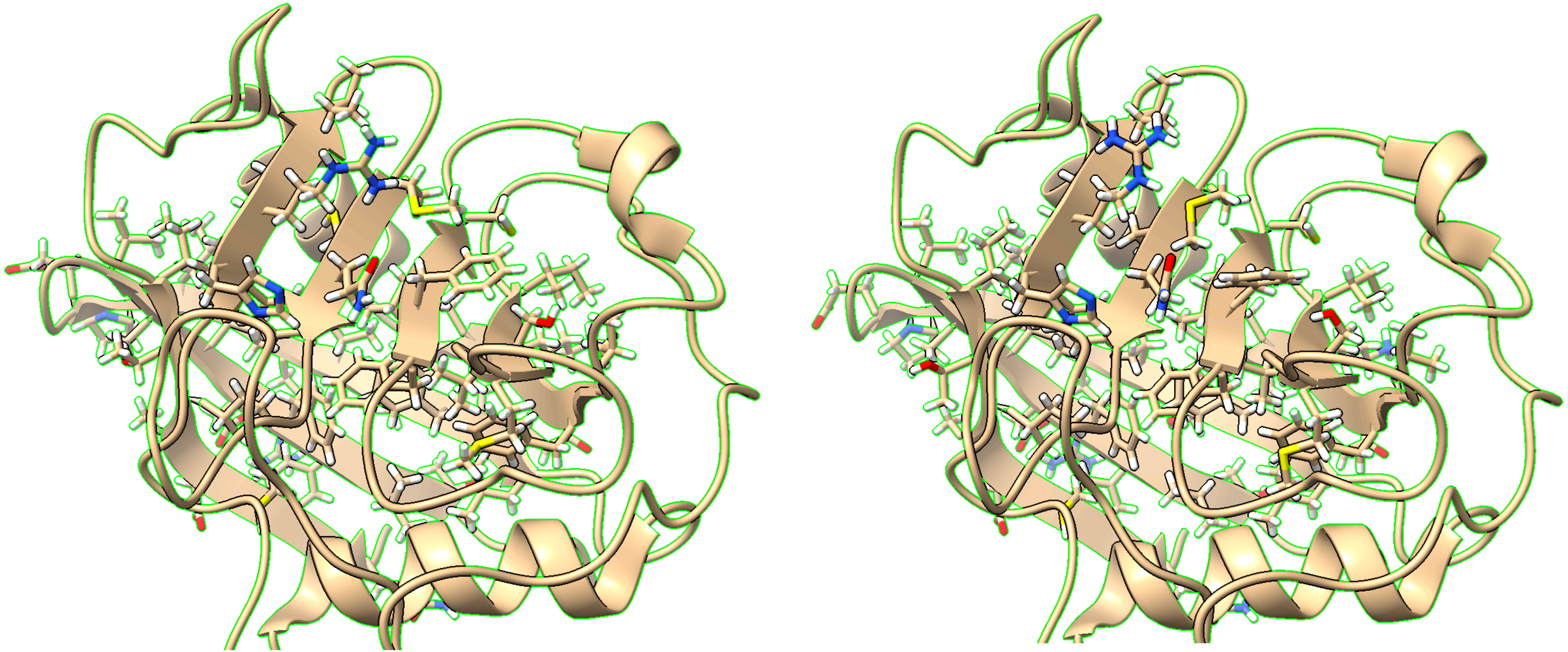
But bound to a drug

1Y57: Complete



Active state, bound to a drug

In this Cyclophilin A structure 22 aminoacids have alternative conformations identified in the electron density (3K0N)



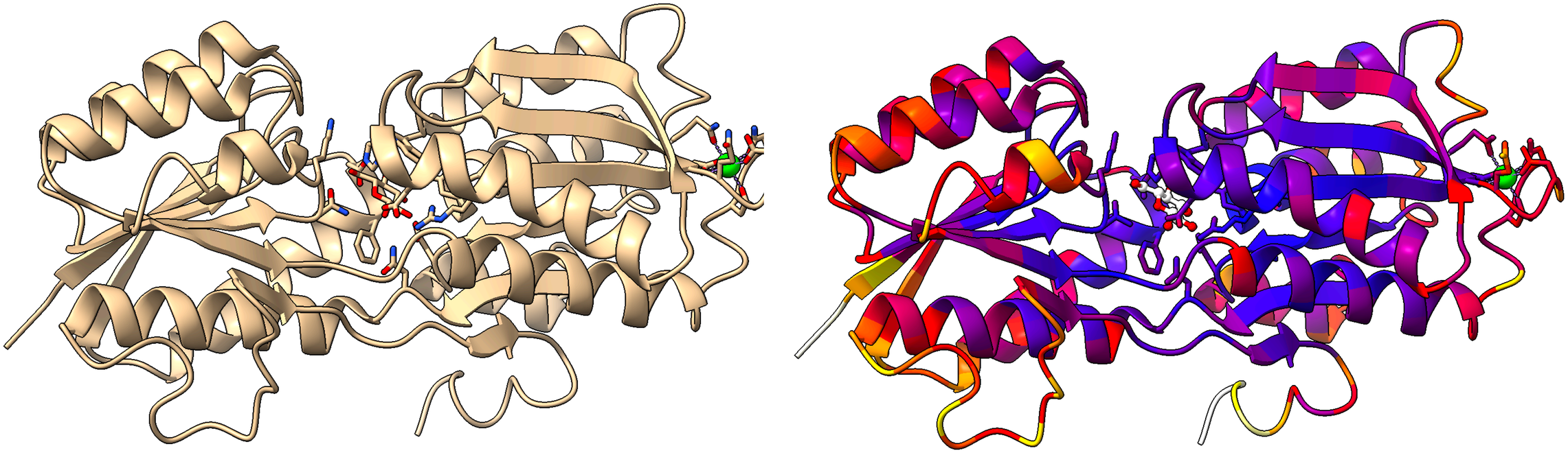
1.Fraser, J. S. *et al*. Hidden alternative structures of proline isomerase essential for catalysis. *Nature* **462**, 669–673 (2009).

What does X-Ray crystallography tell us about molecules motion?

4. All atoms assigned with the density are associated with a B-Factor (Debye-Waller factor, Temperature factor, atomic displacement parameter). This number reports in part about the fluctuation of the atom in the crystal, but it is also the results of a number of other properties. Nonetheless, even if not to be used quantitatively, it can provide indications of different extent of protein motion in crystal, that may be associated to a similar motion in solution

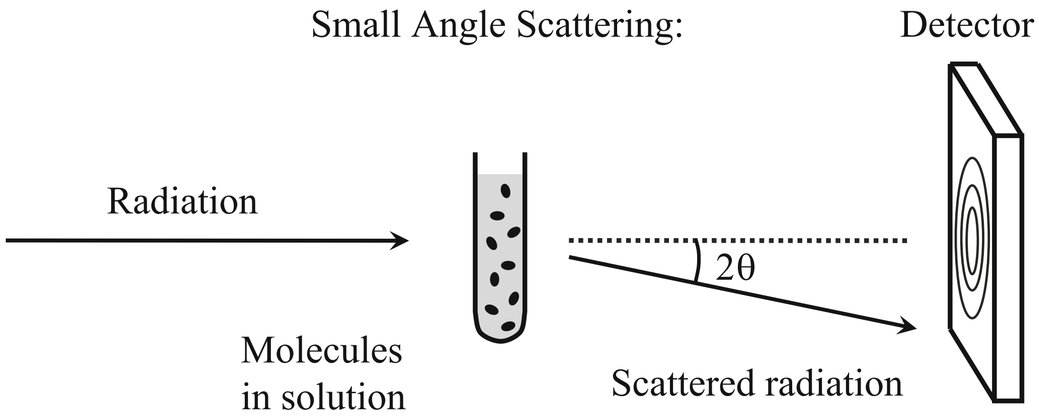
Sun, Z., Liu, Q., Qu, G., Feng, Y. & Reetz, M. T. Utility of B-Factors in Protein Science: Interpreting Rigidity, Flexibility, and Internal Motion and Engineering Thermostability. *Chem. Rev.* **119**, 1626–1665 (2019).

Example: B-Factors



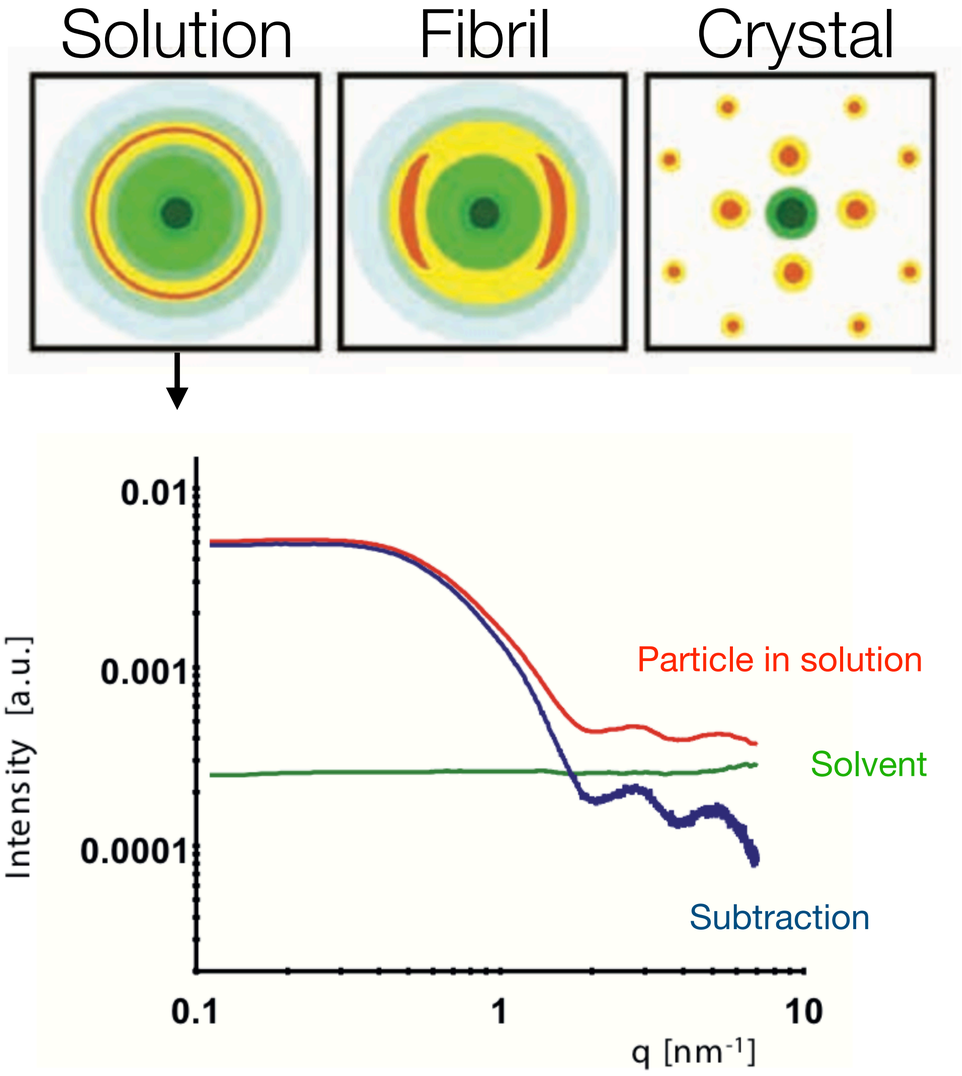
Lighter colours highlight more noisy regions as indentified by B-Factors: 2GBP

X-rays in solution: small angle X-ray scattering (SAXS)



$$I_{\mathrm{m}}(q) = \sum_{j=1}^{N_A} \sum_{j=1}^{N_A} f_i(q) f_j(q) \frac{\sin(q d_{ij})}{q d_{ij}} .$$

The SAXS intensity for a diluite sample is the sum of the atomic composition *f(q)* and their relative distance *d*



X-rays in solution: small angle X-ray scattering (SAXS)

$$I_{\mathrm{m}}(q) = \sum_{j=1}^{N_A} \sum_{j=1}^{N_A} f_i(q) f_j(q) \frac{\sin(q d_{ij})}{q d_{ij}}.$$

Given a protein structure it is possible to calculate its theoretical intensity from the atomic positions, then it is also possible to subtract the effect of bulk water

This is useful to dock proteins together in search of quaternary structure, but also to validate the presence of high-order structures derived from crystals (e.g. is a dimeric structure observed in crystal the result of the crystallisation process or a true dimer already in solution?)

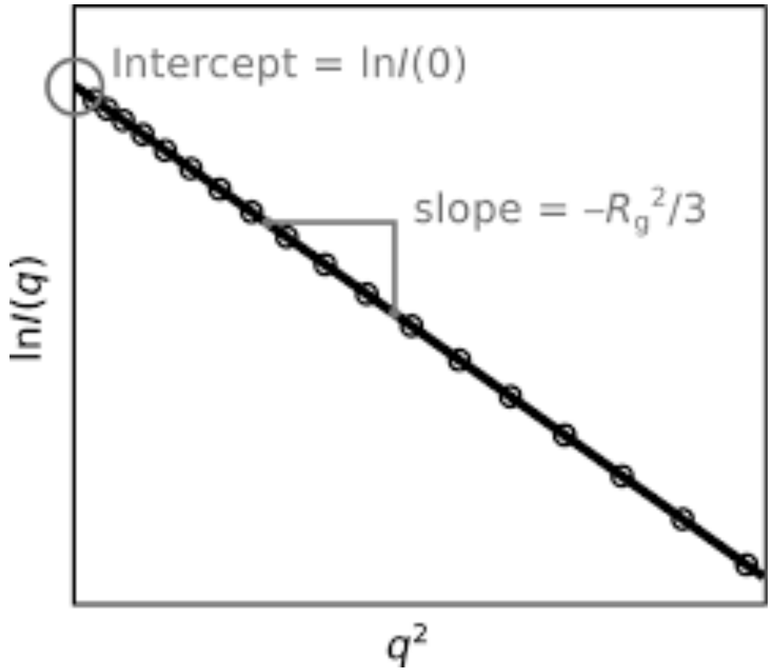
SAXS can be measured for molecules of any size, with no limitations about the extent of their dynamics.

SAXS for intrinsically disordered proteins

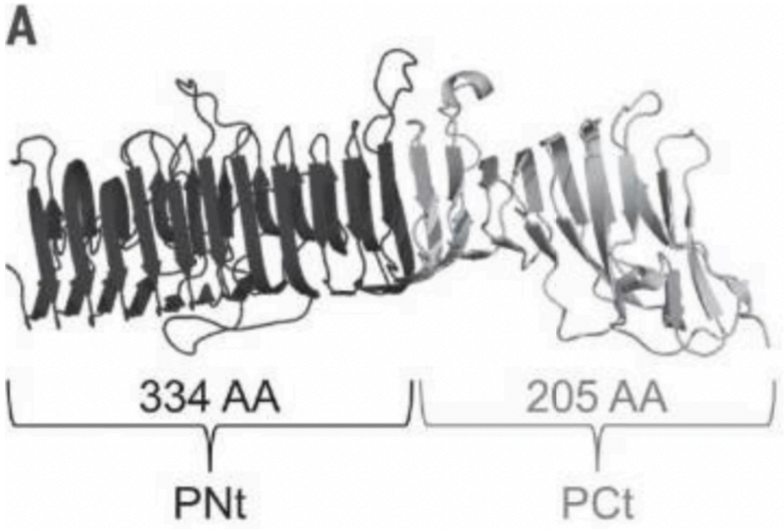
$$I(s) = I(0)e^{\frac{-s^2R_g^2}{3}}$$

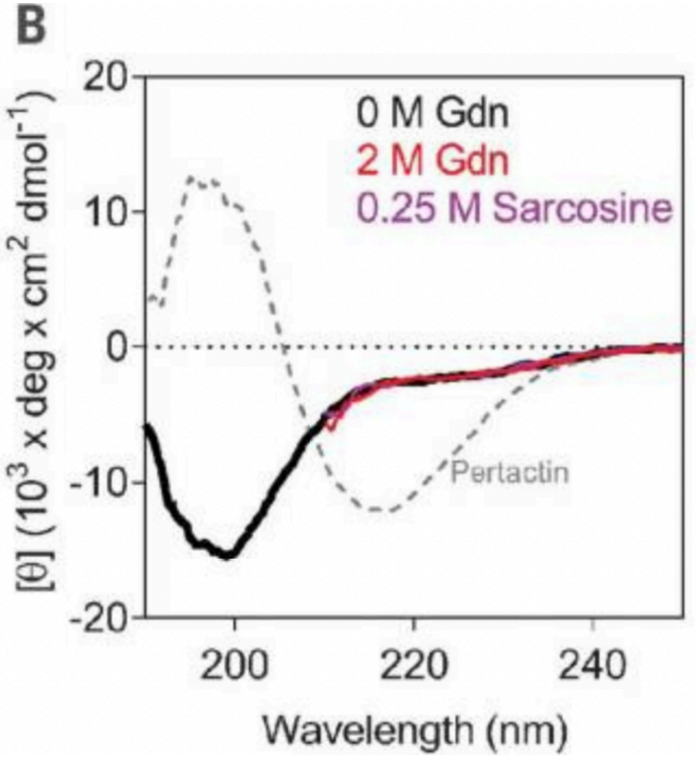
SAXS at very small angles provides information about the size of a system in solution in terms of the radius of gyration. The log of SAXS intensity is proportional to the radius (Guinier relation)

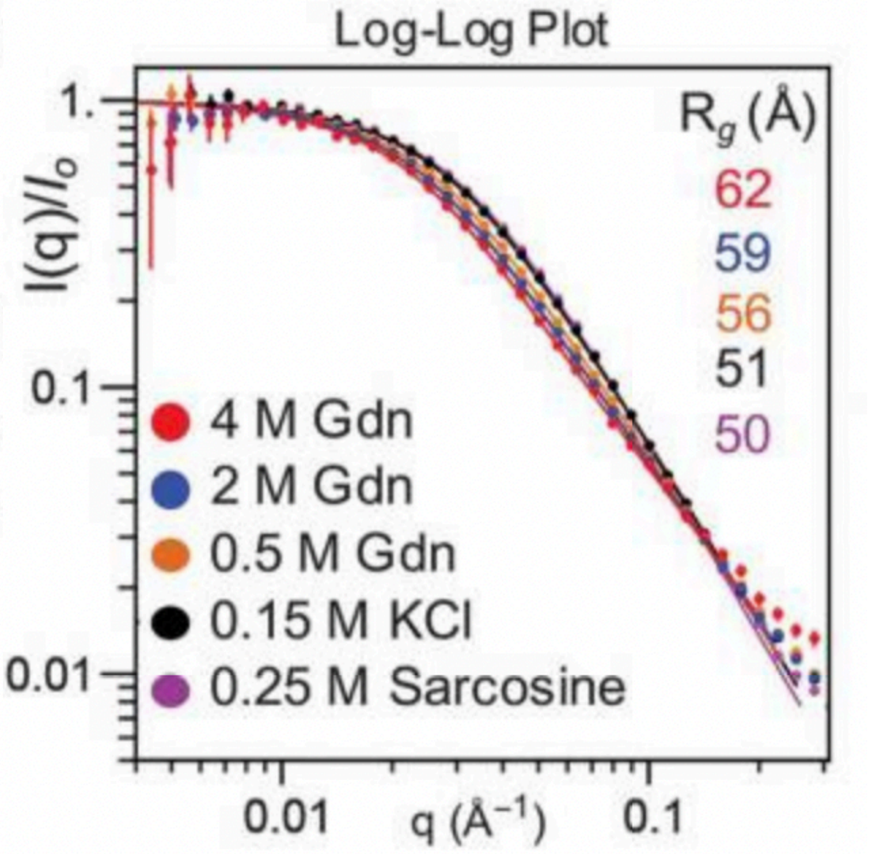
This is useful to interrogate about the oligomeric state of a folded protein but also to detect changes in the conformations explored by intrinsically disordered proteins as a consequence of the solution conditions (solvent composition, temperature, concentration, partners, …)



Example: effect of denaturant on the unfolded state of a protein







PNt is disordered when alone and its size depends on the solution conditions

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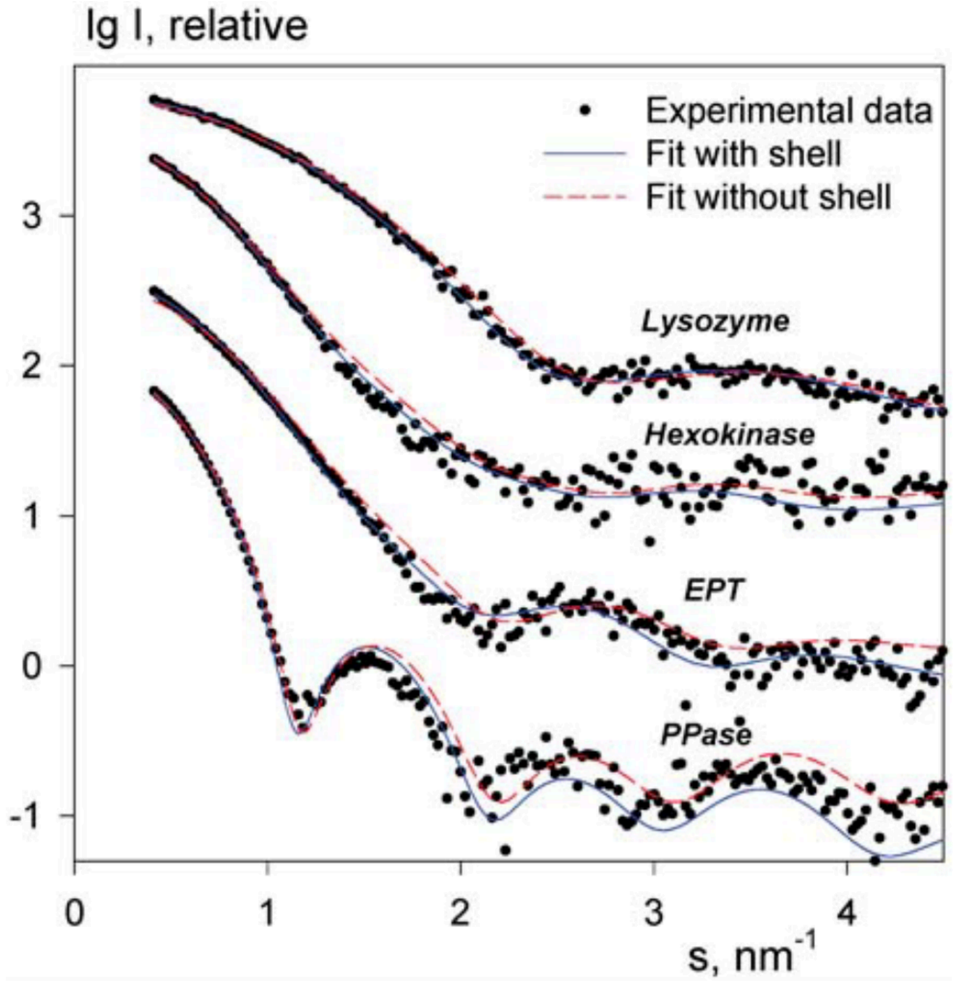
Riback, J. A. *et al*. Innovative scattering analysis shows that hydrophobic disordered proteins are expanded in water. *Science* **358**, 238–241 (2017).

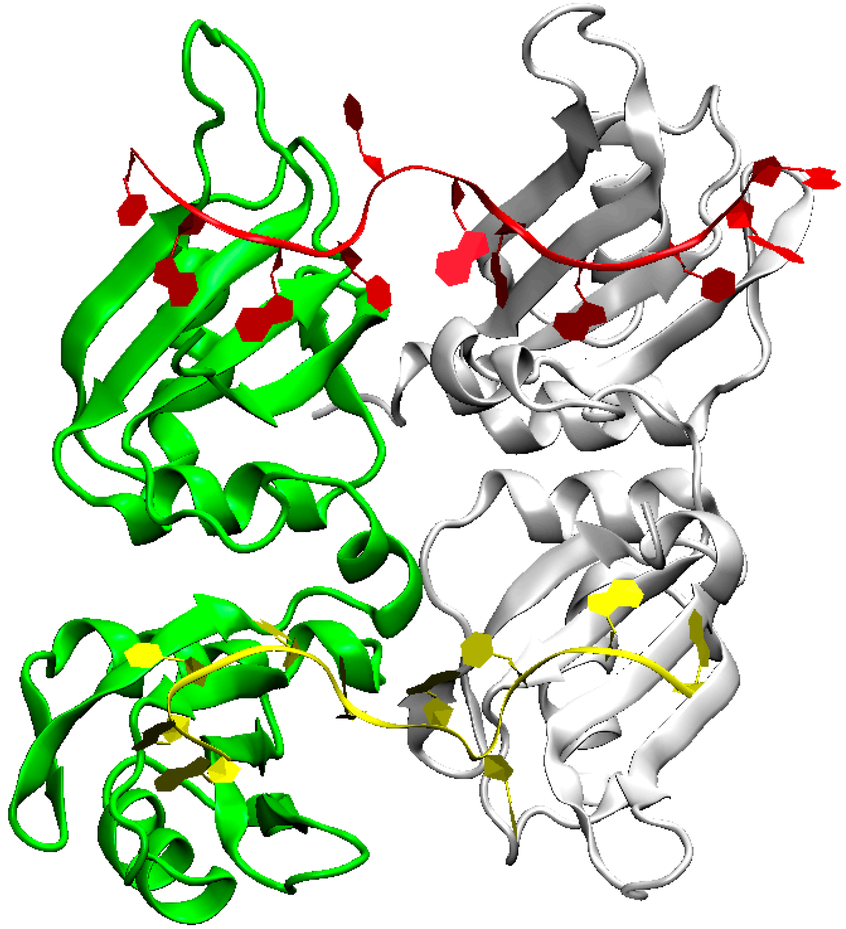
SAXS: testing the validity X-rays structures in solution



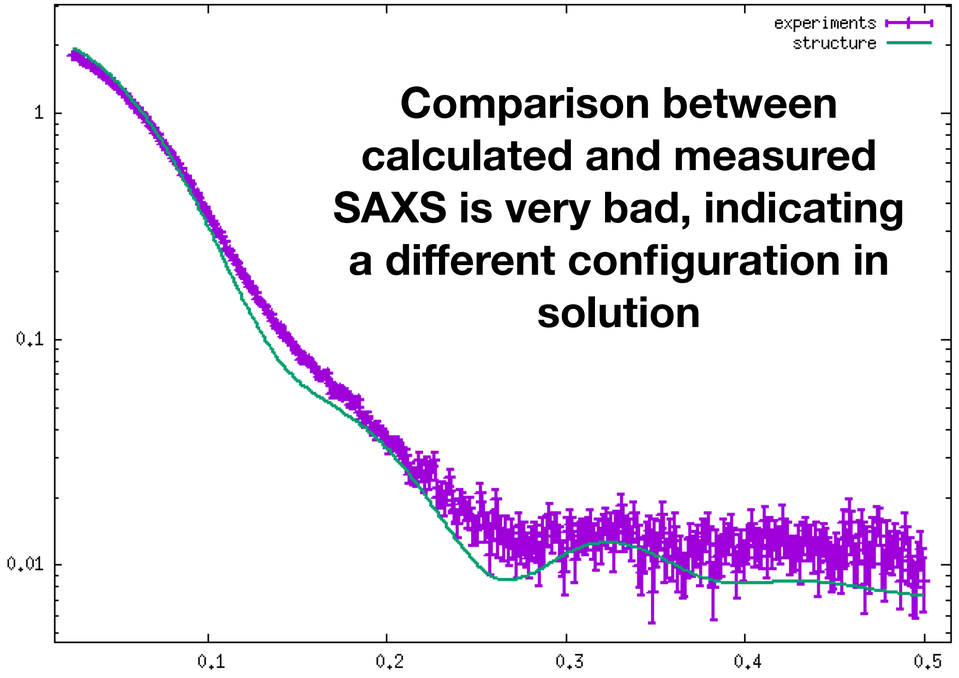
For many proteins, the comparison between the SAXS calculated from their crystal structure and the experimental data is very good, indicating that the structure is representative for the behaviour of the protein in solution.

But this is not always the case, for example for cases of multi-domain proteins with flexible linkers

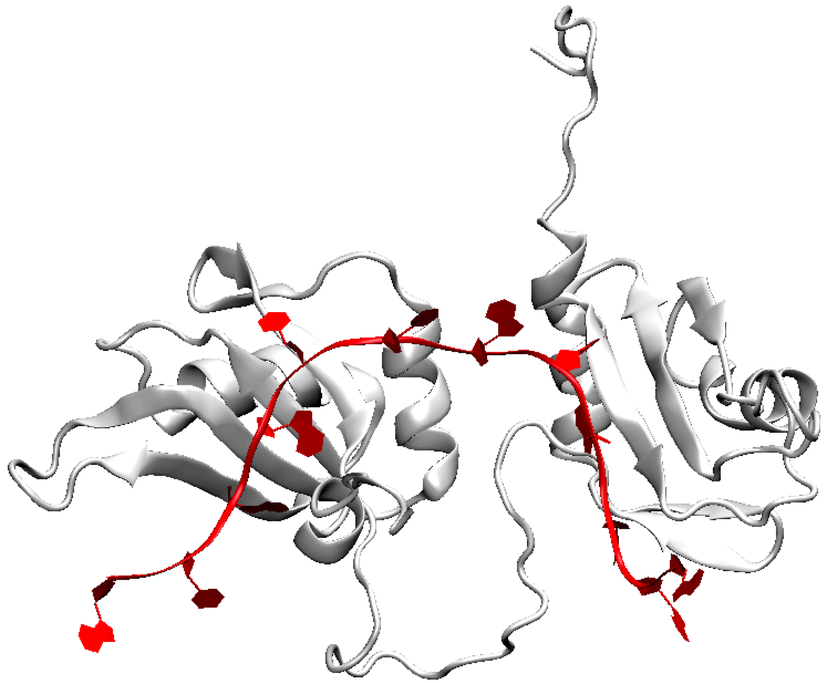
SAXS: testing the validity X-rays structures in solution



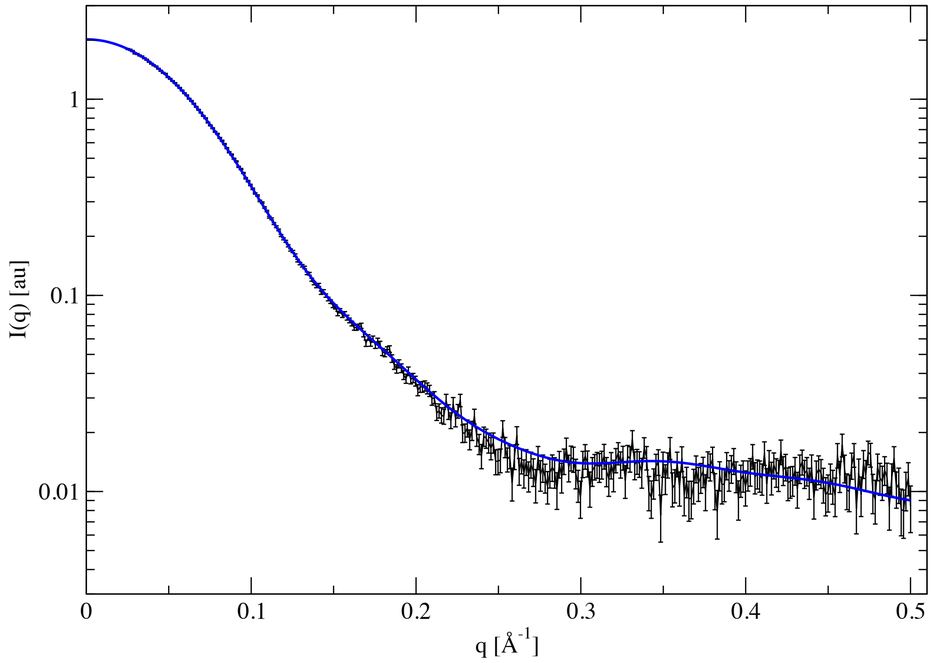
Crystal Structure of the hnRNP A1 protein (RNA binding protein) bound to a short sequence of a pri-miRNA. The structure suggest that a dimeric protein is bound transversally to two miRNA molecules.



SAXS: testing the validity X-rays structures in solution



A structure of a monomeric protein bound to a single RNA fits the SAXS data perfectly:



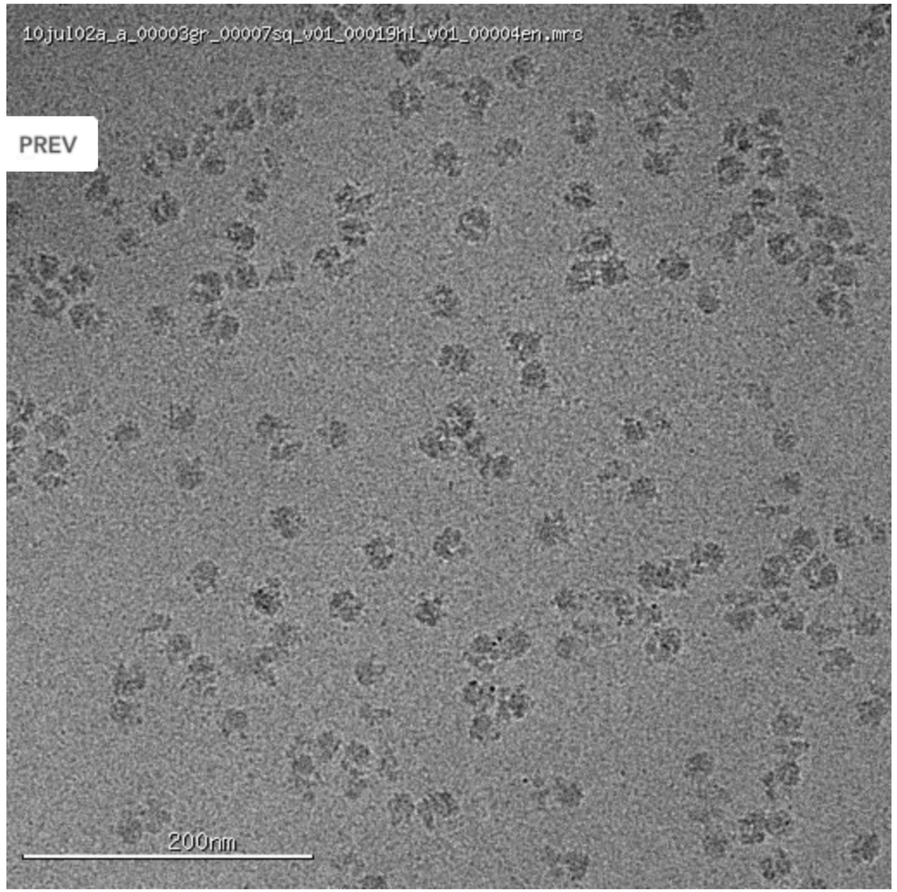
Kooshapur, H. *et al*. Structural basis for terminal loop recognition and stimulation of pri-miRNA-18a processing by hnRNP A1. *Nat*. *Commun*. **9**, 2479 (2018).

So how do we “take pictures” of molecules? 2. Cryo-Electron Microscopy

Issues are signal/noise, small size, motion on many time scales

SOLUTION: Solution is vitrified by liquid nitrogen on a plate: essentially all the molecules are on a plane, and electron (very few) are sent through the sample to avoid molecular motions so we get clear 2D shadows

See also:
https://www.youtube.com/watch?v=BJKkC0W-6Qk



Ribosomes.
Image 2 of 2

CLOSE

What does cryo-EM tell us about molecules motion?

The key idea of cryo-EM is that of removing protein motion by freezing the system. Both the motion of the different molecules, that are trapped in the vitrified solution, but also the internal motion.

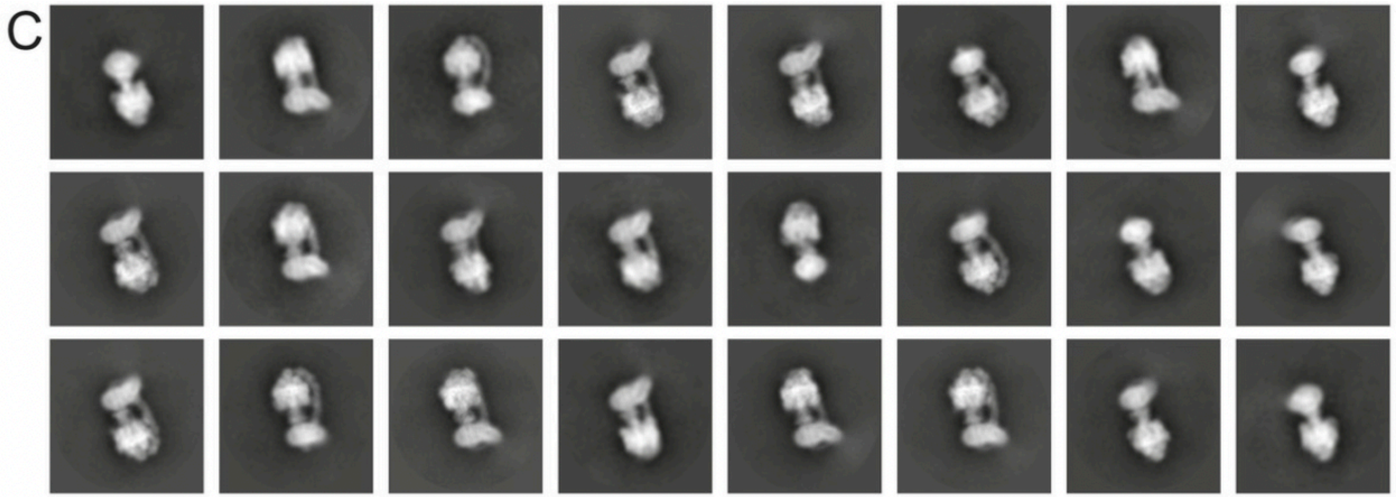
At odds with crystallography here different copies of the molecule can be trapped in different conformations. But as a consequence it may be difficult to accumulate enough coherent data to determine a structure.

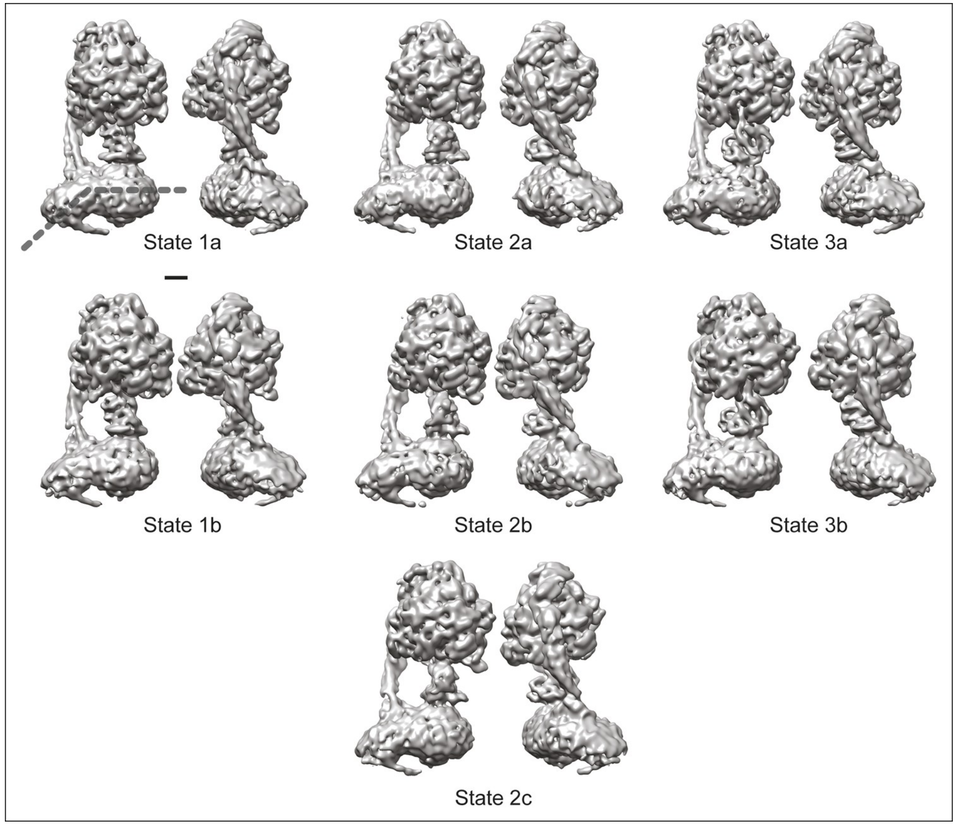
Cryo-EM can be used to identify molecules in multiple conformations

1. Shadows with the same shape (thousands) are clustered and averaged to increase the signal. Image recognition techniques are used to speed up the process.
2. Averaged 2D images are then organised in clusters: are the 2D images representing the same conformation?
3. All the images that are associated to the same conformation are then used to obtain a 3D density map in which it is then possible to build an atomic model

If the molecule populates too many conformations, or if regions of the molecule are too flexible they are invisibile because it is not possible to accumulate enough 2D images to improve the signal to noise ratio. Different classes may result in different RESOLUTION both for different regions of same conformation as well as for different conformations.

Example: Structure and conformational states of the bovine mitochondrial ATP synthase by cryo-EM

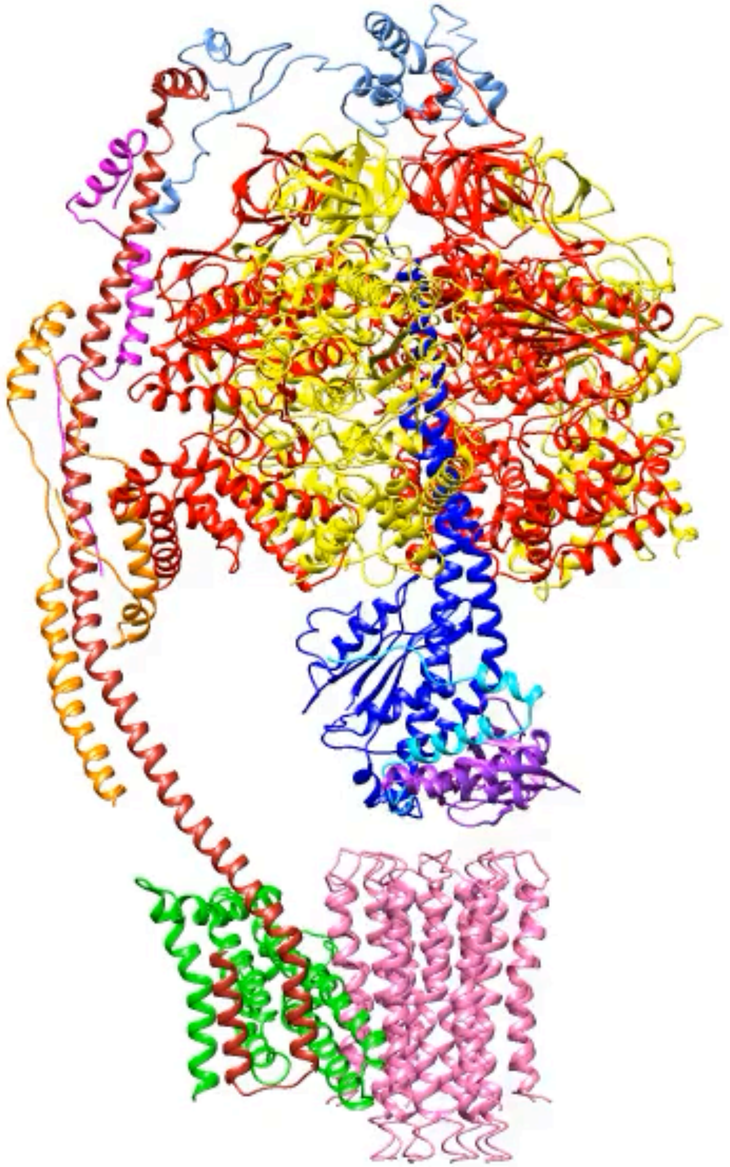




Adenosine triphosphate (ATP), the chemical energy currency of biology, is synthesized in eukaryotic cells primarily by the mitochondrial ATP synthase. ATP synthases operate by a rotary catalytic mechanism where proton translocation through the membrane-inserted FO region is coupled to ATP synthesis in the catalytic F1 region via rotation of a central rotor subcomplex.

This is an interpolation of three states to render a movie

It is not necessarily how the conformational change happens

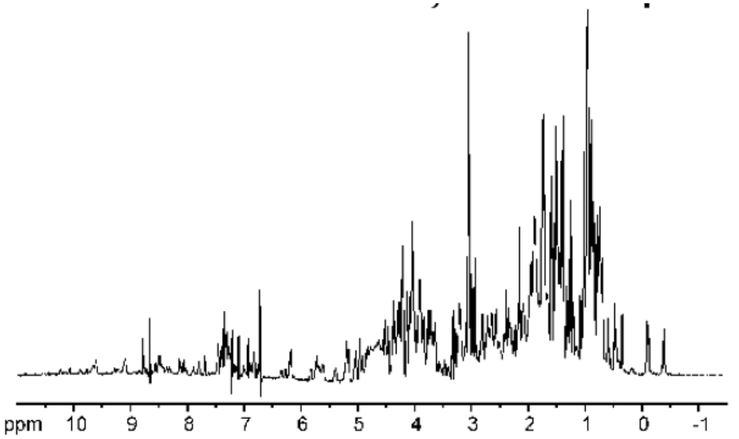


https://elifesciences.org/articles/10180#abstract

So how do we “take pictures” of molecules? 3. Nuclear Magnetic Resonance Spectroscopy

Issues are signal/noise, small size, motion on many time scales

SOLUTION: We employ the radio frequency absorption and emission properties of nuclei in presence of a magnetic field. In this way we can look at the signal of the same atom in all the molecules.



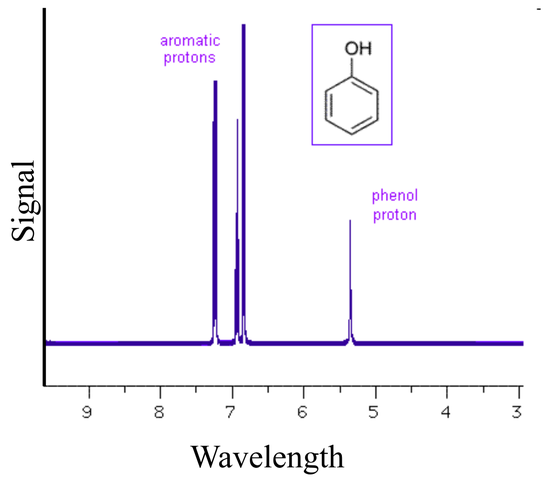
Here the signal to noise ratio is determined by the protein concentration and the size of the molecules, where the larger the molecule the weaker the signal (usually)

Principles of NMR spectroscopy

- **Principles:**
  - A strong **external electric field** applied by the NMR machine to the sample results in **spatial alignment** of protein atoms with nuclear spin (e.g. ^{1}H, ^{13}C , ^{15}N)
  - An **energy pulse** transiently disturbs this alignment
  - The nuclei revert to their original state while **emitting radio waves** that are sensed by the NMR machine
  - The waves are different for each atoms, and affected by their atom neighbors (**chemical shift**)
  - By using the known chemical shifts of nuclei at different chemical environments, **the structure of the protein can be deciphered** from its NMR spectrum

Introduction to Proteins, Kessel and Ben-Tal, 2018

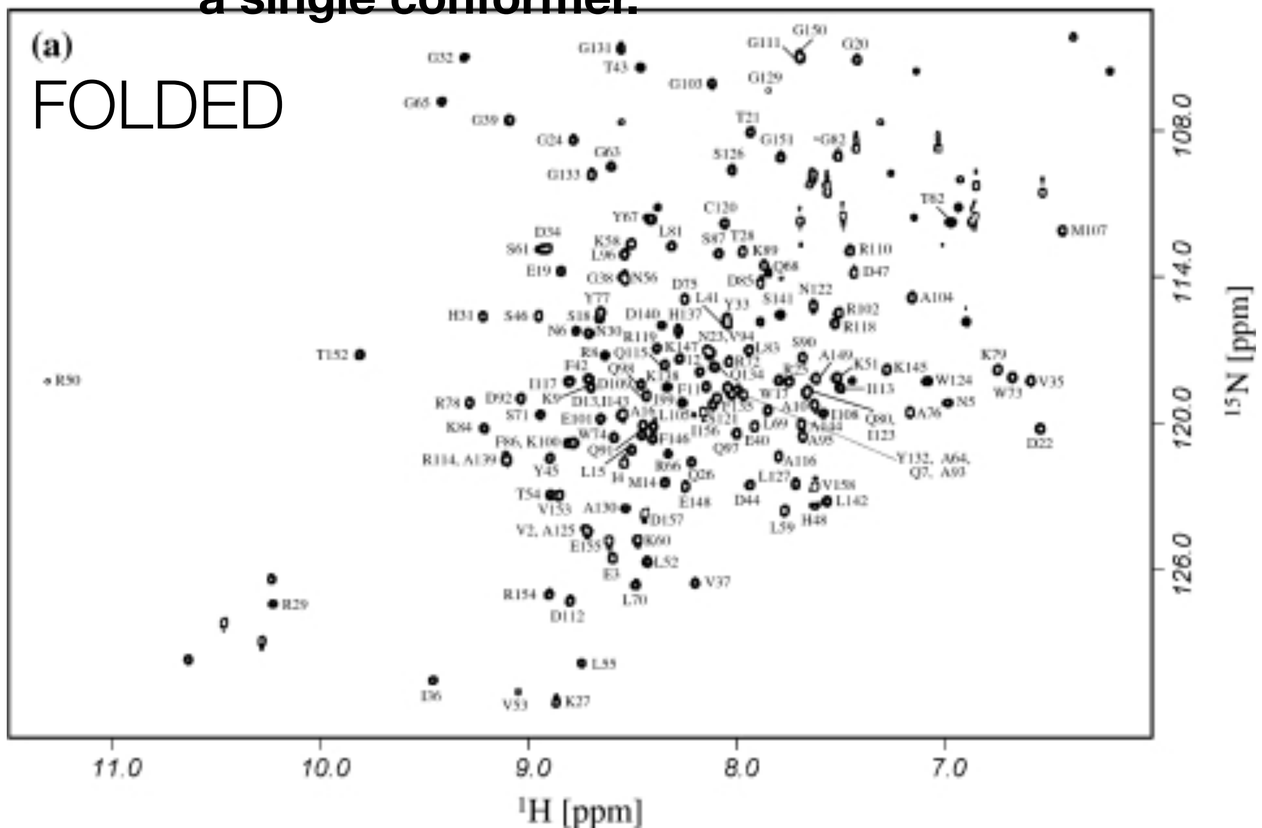
- 1D NMR spectrum of protons from different locations in a phenol molecule:

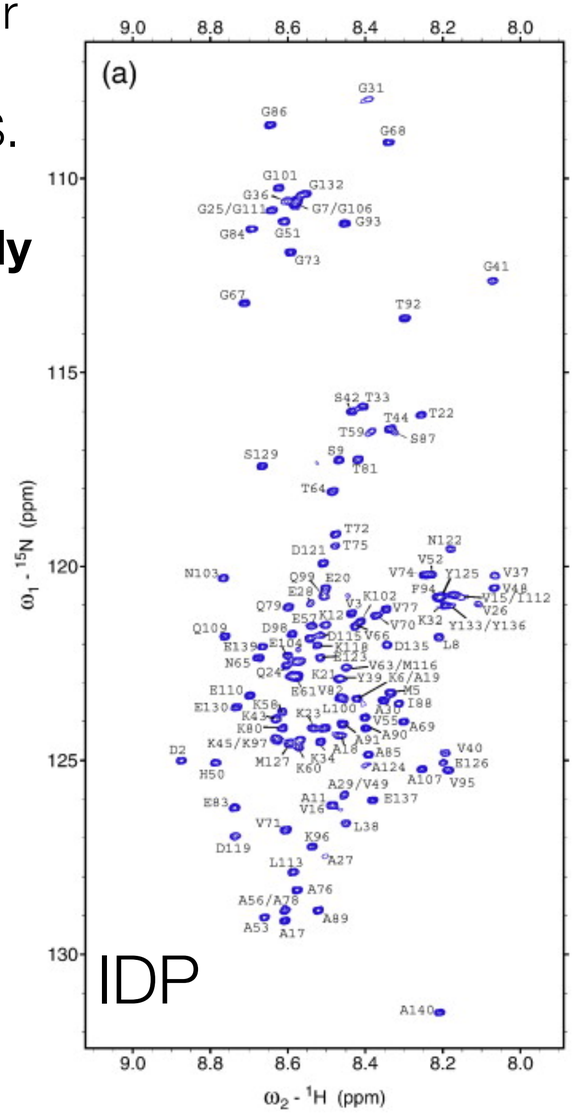


What does NMR tell us about molecules motion?

With NMR we can observe molecules in solution, without posing any constraint on their intermolecular and internal motion, apart from keeping a relatively high concentration. Indeed chemical shifts can be observed equally well for folded proteins as well as IDPS.

At the same time other NMR measurements typically used for structure determination, like NOE, do work only if the protein in solution populates mostly a single conformer.





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NMR and X-ray structure are generally the same indicating that the crystal structure is representative of in solution.

Volume 206, Issue 4, 20 April 1989, Pages 677-687

Comparison of the high-resolution structures of the α-amylase inhibitor tendamistat determined by nuclear magnetic resonance in solution and by X-ray diffraction in single crystals

Martin Billeter [1], Allen D. Kline [1 †], Werner Braun [1], Robert Huber [2], Kurt Wüthrich [1]

The three-dimensional structure of the α-amylase inhibitor Tendamistat determined by nuclear magnetic resonance in aqueous solution is compared with the Tendamistat crystal structure refined at 2·0 Å resolution. Between the two independently obtained structures the root-mean-square distances are 1·05 Å for the backbone atoms N, Cα and C′, 1·25 Å for the backbone and the interior side-chains, and 1·84 Å for all heavy atoms. These numbers show that the interior of the molecule is nearly identical in the two states. Near the protein surface a small number of local differences between the two structures were identified. In most surface areas the solution structure appears more disordered than the crystal structure, with the exception of Tyr15, which was not observed in the X-ray diffraction.

NMR structures can also be determined in cell

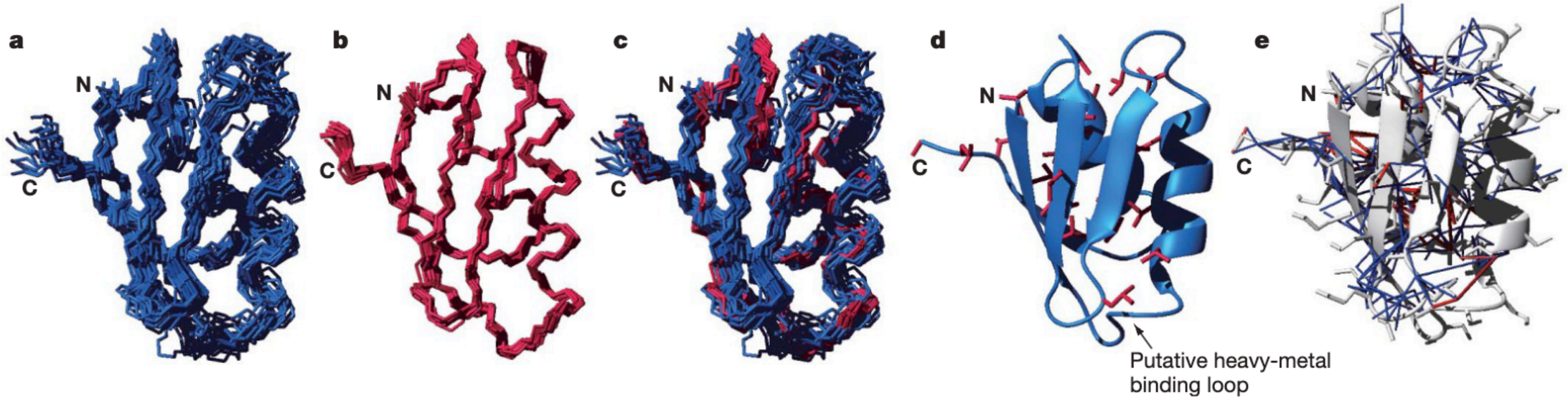


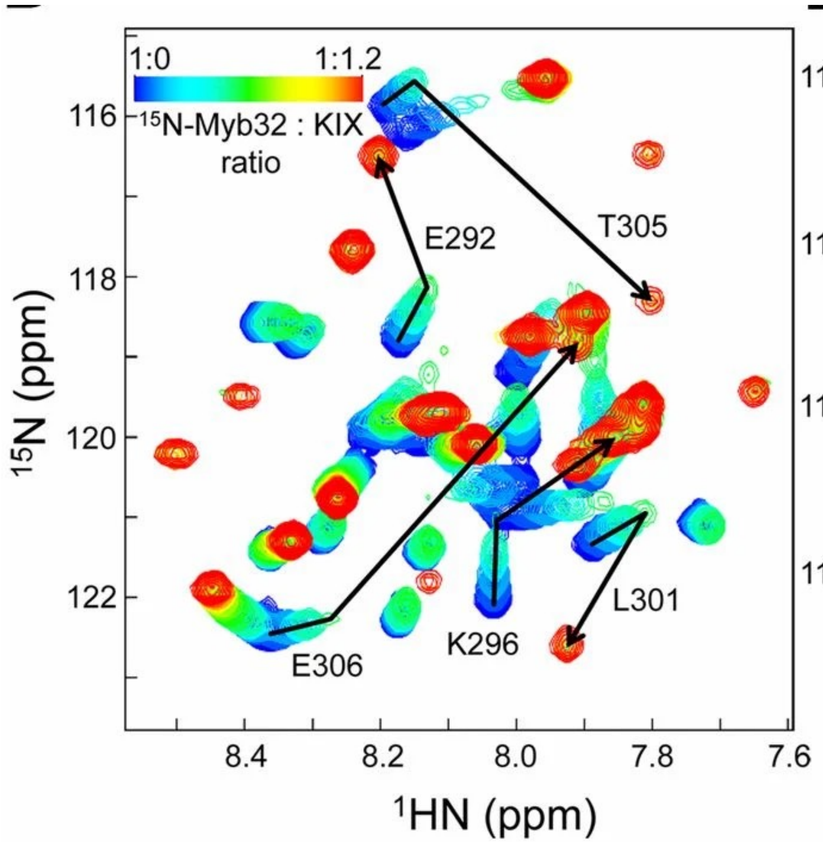
Figure 4 | NMR solution structure of TTHA1718 in living *E. coli* cells. **a**, A superposition of the 20 final structures of TTHA1718 in living *E. coli* cells, showing the backbone (N, Cα, C′) atoms. **b**, A superposition of the 20 final structures of purified TTHA1718 *in vitro*. **c**, A comparison of TTHA1718 structures in living *E. coli* cells and *in vitro*. The best fit superposition of backbone (N, Cα, C′) atoms of the two conformational ensembles are shown with the same colour code in **a** and **b**. **d**, Secondary structure of TTHA1718 in living *E. coli* cells. The side chains of Ala, Leu and Val residues, the methyl groups of which were labelled with $^{1}H/^{13}C$, are shown in red. **e**, Distance restraints derived from methyl-group-correlated and other NOEs are represented in the ribbon model with red and blue lines, respectively.

Protein structure determination in living cells by in-cell NMR spectroscopy

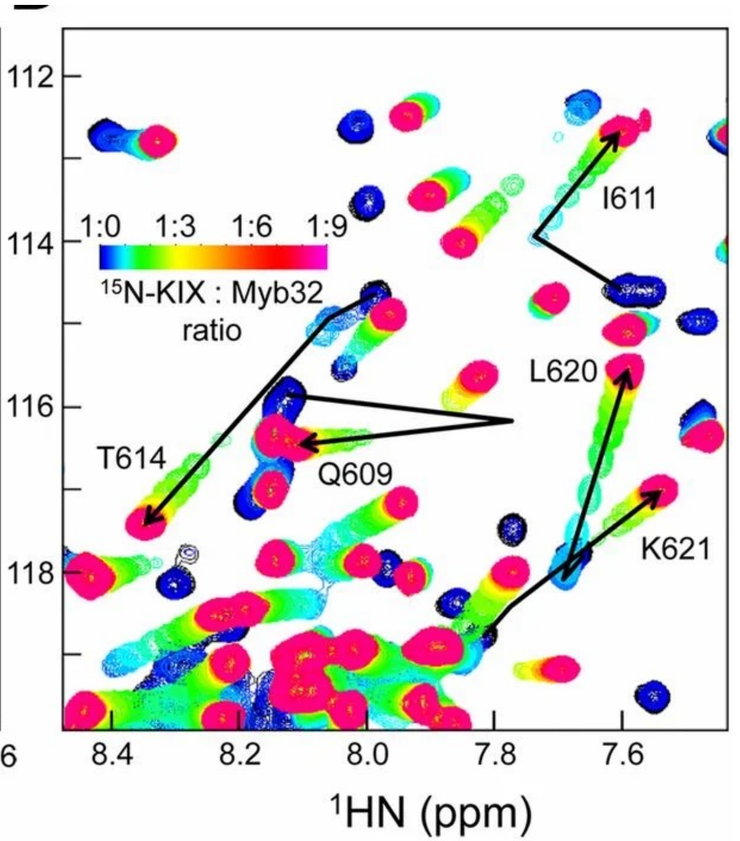
Daisuke Sakakibara[1,2]*, Atsuko Sasaki[1,2]*, Teppei Ikeya[1,3]*, Junpei Hamatsu[1,2], Tomomi Hanashima[1], Masaki Mishima[1,2], Masatoshi Yoshimasu[4], Nobuhiro Hayashi[5]†, Tsutomu Mikawa[6], Markus Wälchli[7], Brian O. Smith[8], Masahiro Shirakawa[2,9], Peter Güntert[1,3,10] & Yutaka Ito[1,2,6]

What does NMR tell us about molecules motion?

But chemical shifts, as well as other NMR experiments, can be used to follow conformational transition resulting from changes in the solution conditions as well as from interactions:



15N-labeled Myb32 showing chemical shift changes upon titration with KIX.

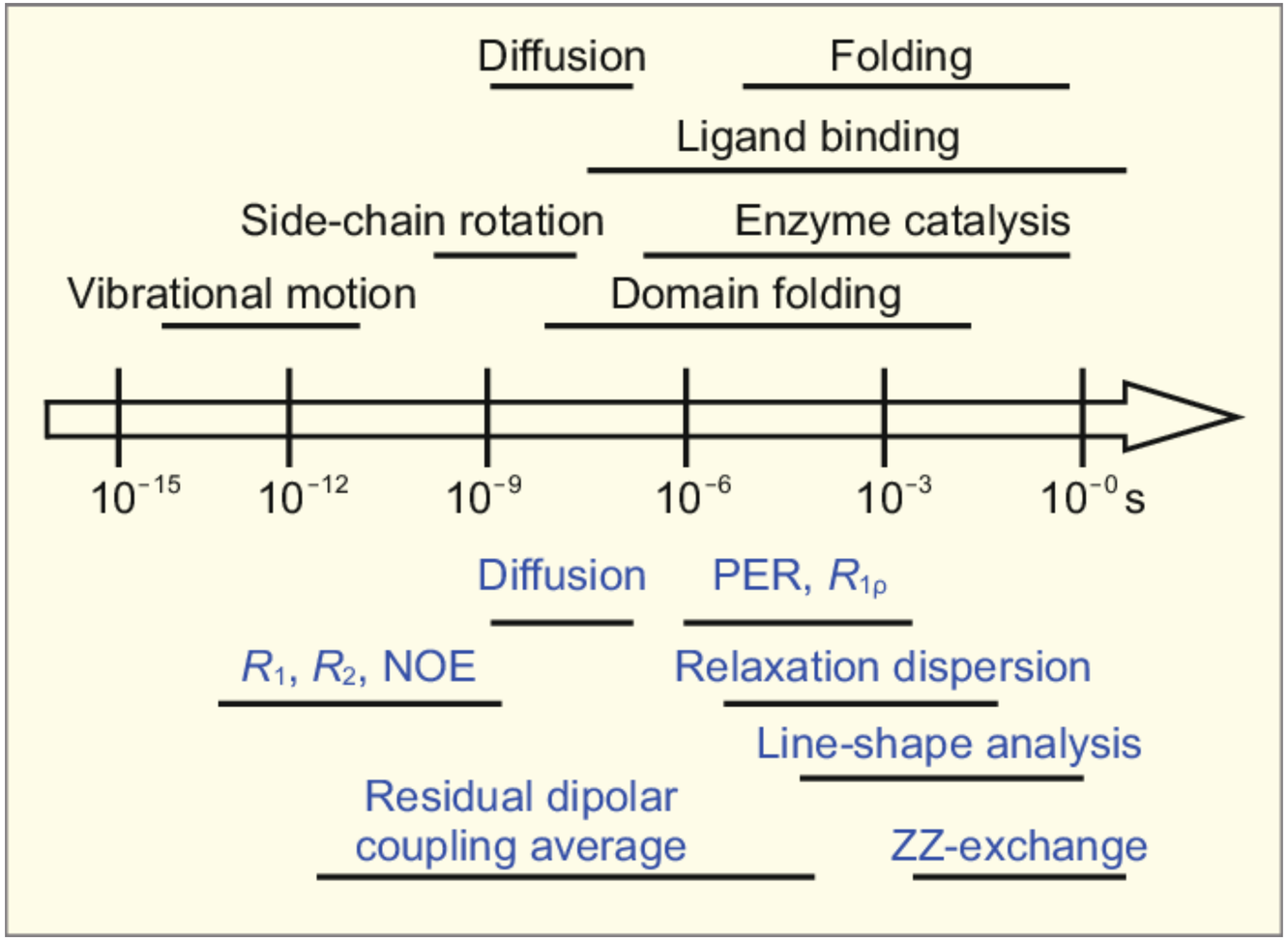


15N-labeled KIX showing chemical shift changes upon titration with Myb32.

Arai, M., Sugase, K., Dyson, H. J. & Wright, P. E. Conformational propensities of intrinsically disordered proteins influence the mechanism of binding and folding. *Proc. Natl. Acad. Sci.* **112**, 9614–9619 (2015).

What does NMR tell us about molecules motion?

But the strength of is that of enabling a large number of different experiments that can probe intra- and inter- molecular interactions focusing on different time-scales



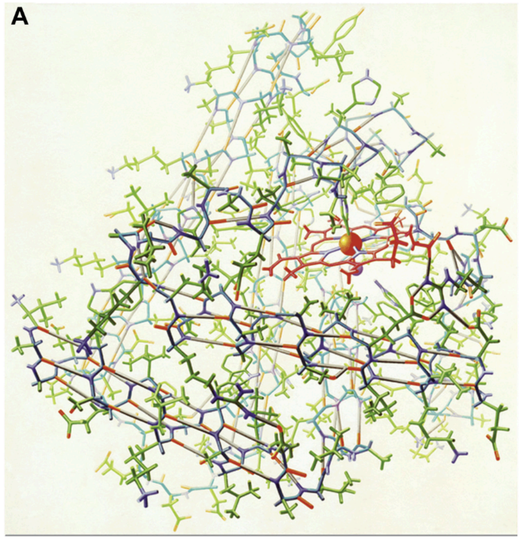
To summarise:

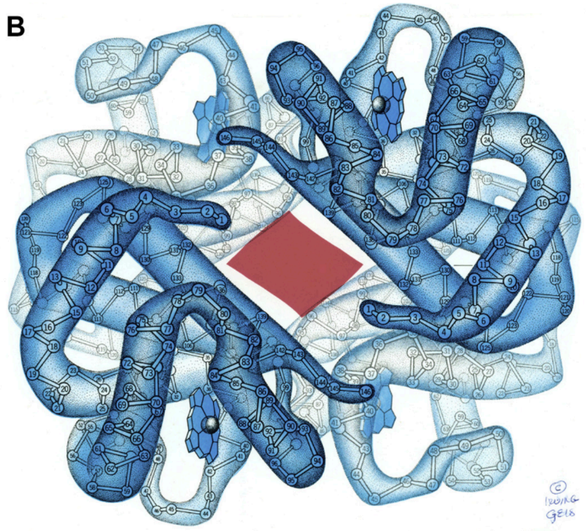
Structural Biology techniques have solved the problem of observing at atomic resolution the world of biomolecules by different strategies. Altogether they allow to appreciate the fact that biomolecules can populate multiple conformations and to characterise some of them. They do not allow to directly observe movements, transitions, and mechanisms at play nor weakly populated conformations that may still be relevant in some conditions.

The computational techniques we will see in the following lectures are designed to fill some of the gaps left by structural biology experiments.

Biomolecular Visualisation

The result of a structure determination experiment is a structure, but in practice this is a collection of relative coordinates. How do you look at them?



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ATOM      3  C   MET A   1      37.035   3.970  16.806  1.00 72.52           C
ATOM      4  O   MET A   1      37.861   3.372  17.506  1.00 73.81           O
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ATOM      7  SD  MET A   1      35.031  -0.302  17.938  1.00 74.07           S
ATOM      8  CE  MET A   1      34.483  -0.461  19.683  1.00 80.55           C
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ATOM     38  CD1 LEU A   5      47.884  11.128  18.668  1.00 18.37           C
ATOM     39  CD2 LEU A   5      46.497  11.561  20.697  1.00 14.10           C
```

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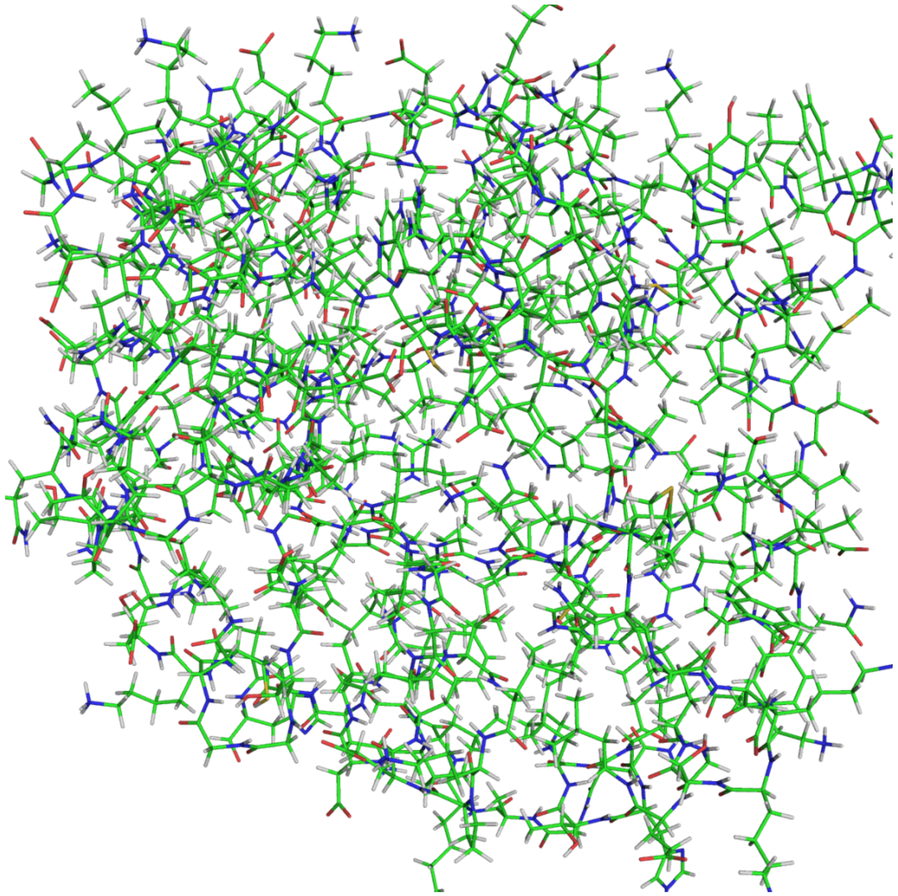
Richardson, J. S., Richardson, D. C. & Goodsell, D. S. Seeing the PDB. *J Biological Chem* **296**, 100742 (2021).

Biomolecular Visualisation

The advent of computers has allowed to dig into the structures and consequently has pushed to define standards to help the rationalisation of the structural features

- **Wireframe (bonds connectivity)**

The covalent bonds in the protein are shown as wires, coloured by the type of atoms they connect. The atoms are not shown.



Introduction to Proteins, Kessel and Ben-Tal, 2018

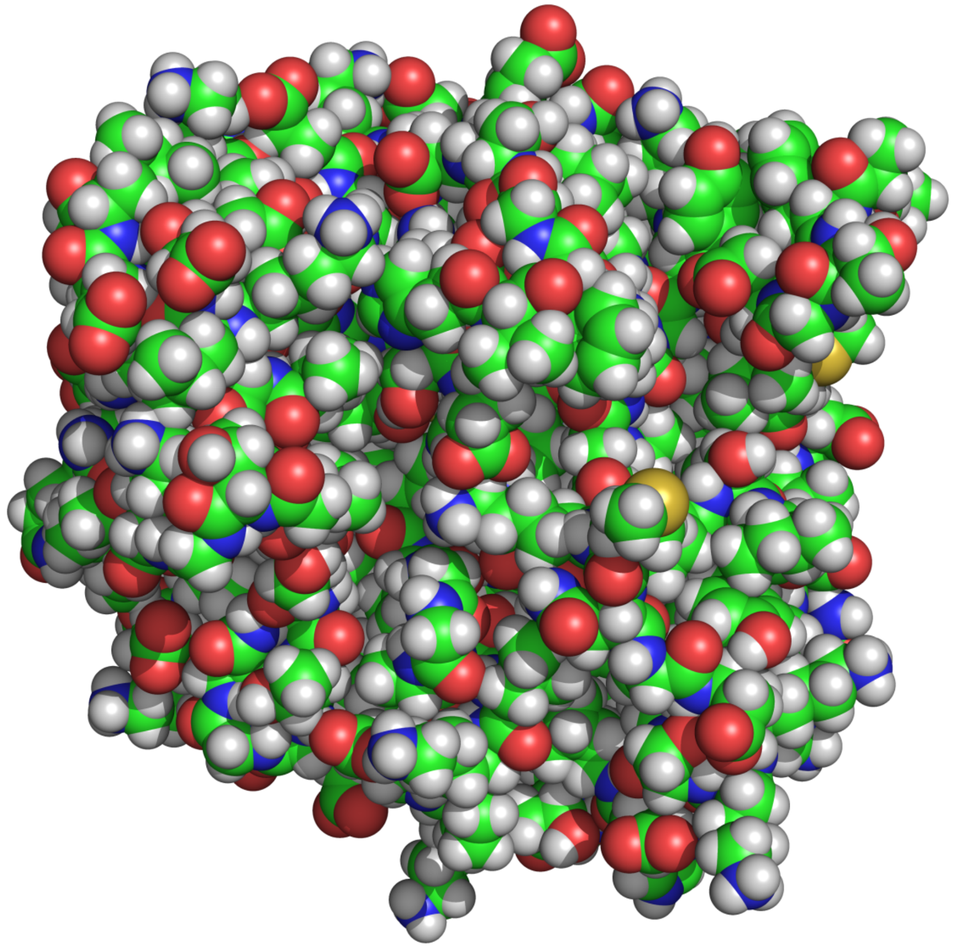
Different graphical representations reveal different properties

The atoms are represented as spheres with a van der Waals radius, and coloured according to the CPK convention

In chemistry, the **CPK coloring** (for Corey–Pauling–Koltun) is a popular color convention for distinguishing atoms of different chemical elements in molecular models.

- White for hydrogen
- Black for carbon
- Blue for nitrogen
- Red for oxygen
- Deep yellow for sulfur
- Purple for phosphorus
- Light, medium, medium dark, and dark green for the halogens (F, Cl, Br, I)
- Silver for metals (Co, Fe, Ni, Cu)

- **Space-fill (general shape, size)**

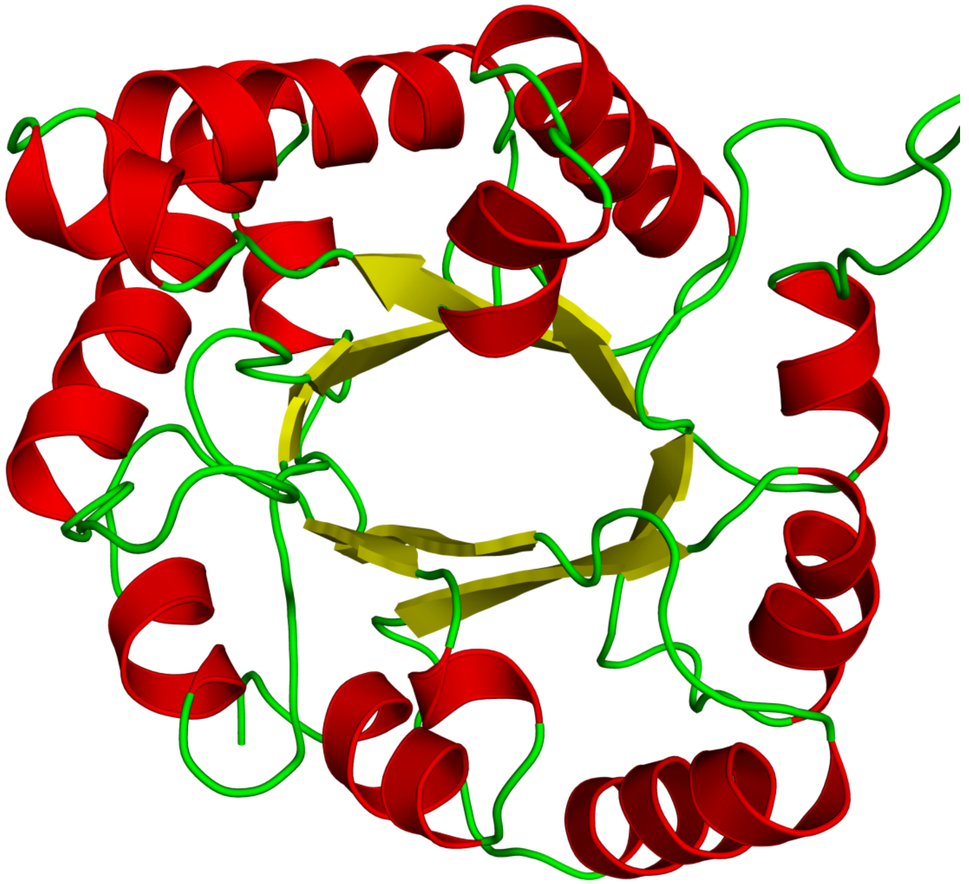


Introduction to Proteins, Kessel and Ben-Tal, 2018

Different graphical representations reveal different properties

- **Ribbon (topology, secondary structures)**

The atoms and bonds of the protein are not shown. Instead, the backbone is represented as a ribbon with different colors, according to the secondary structure of the chain in that region: α-helices are colored in red, β-strands in yellow, and loops in green, as well as the disordered parts of the chain. The shape of the ribbon was calculated as the line going through all the C_α atoms of the protein.

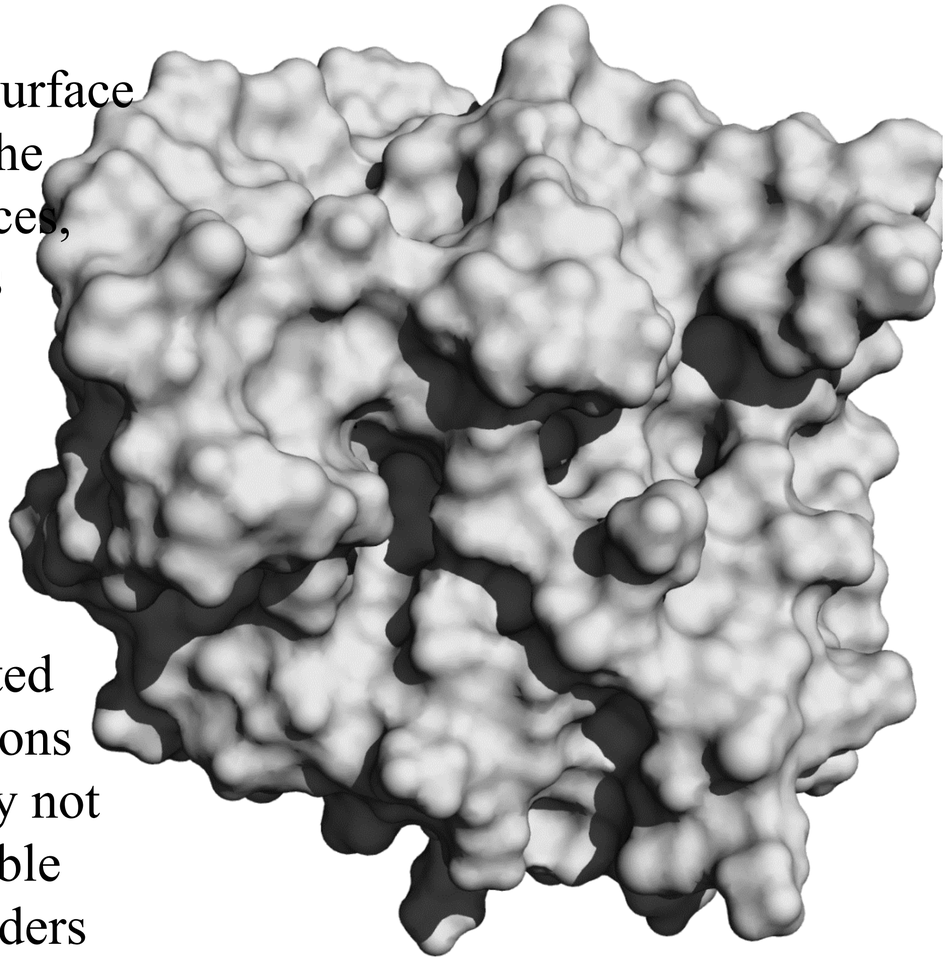


Introduction to Proteins, Kessel and Ben-Tal, 2018

Different graphical representations reveal different properties

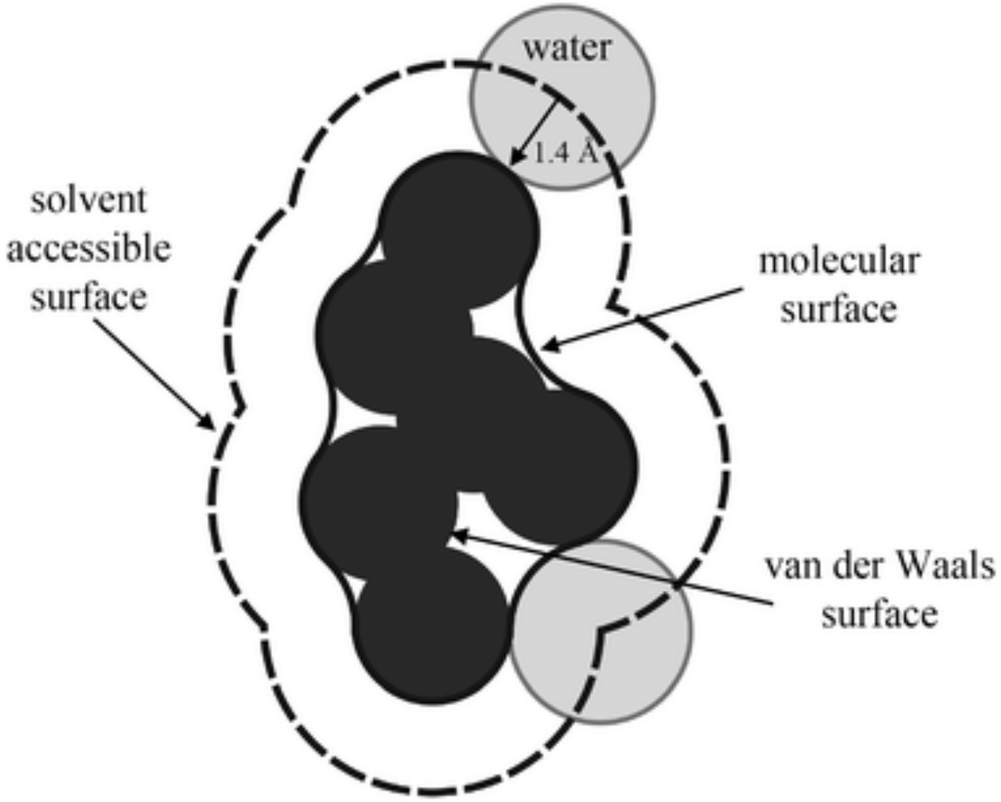
- **Surface (potential binding sites)**

The water-accessible surface is shown, illustrating the indentations and crevices, which may function as binding sites.



Surface can be calculated in different ways. Regions accessible to water may not represent those accessible to larger or smaller binders

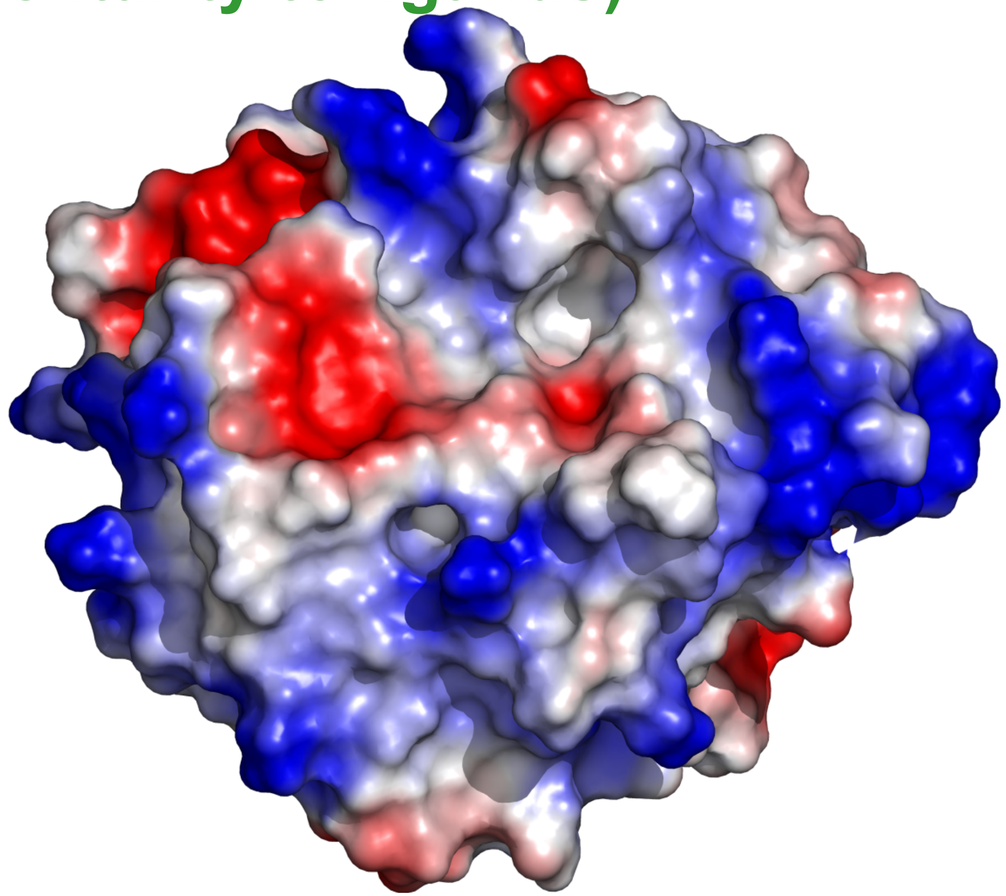
Types of surfaces



Different graphical representations reveal different properties

The figure was produced using PyMol. Negative potentials ($0k_BT/e > F > -60k_BT/e$) are red, positive potentials ($0k_BT/e < F < 60k_BT/e$) are blue, and neutral potentials are white. The electrostatic potential was calculated using APBS.

- **Surface, colored by electrostatic potential (Φ) (complementarity to ligands)**

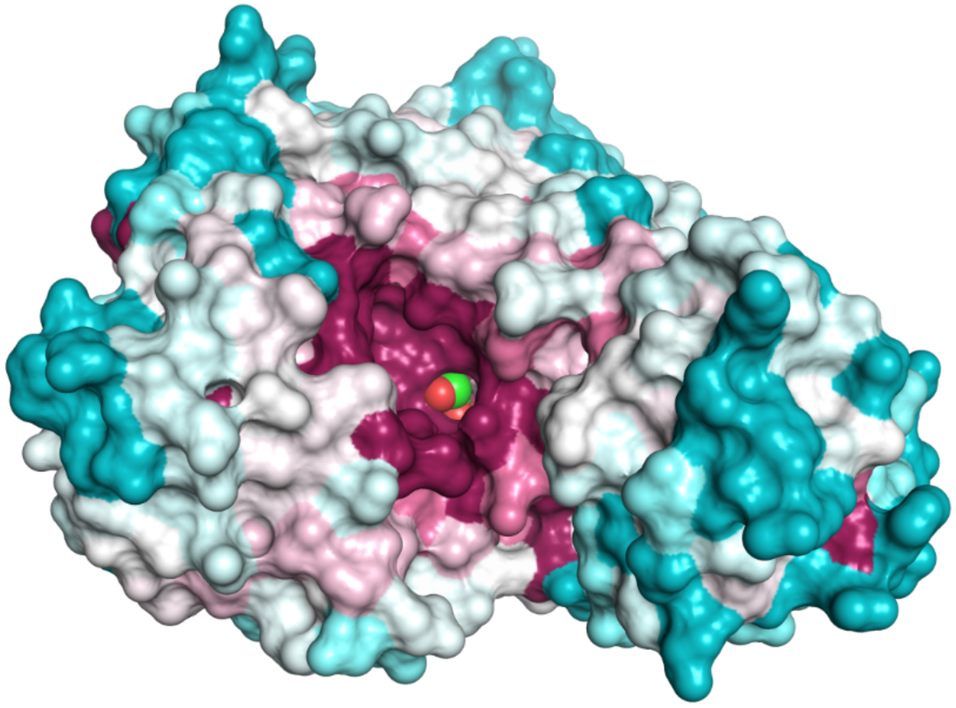


Introduction to Proteins, Kessel and Ben-Tal, 2018

Different graphical representations reveal different properties

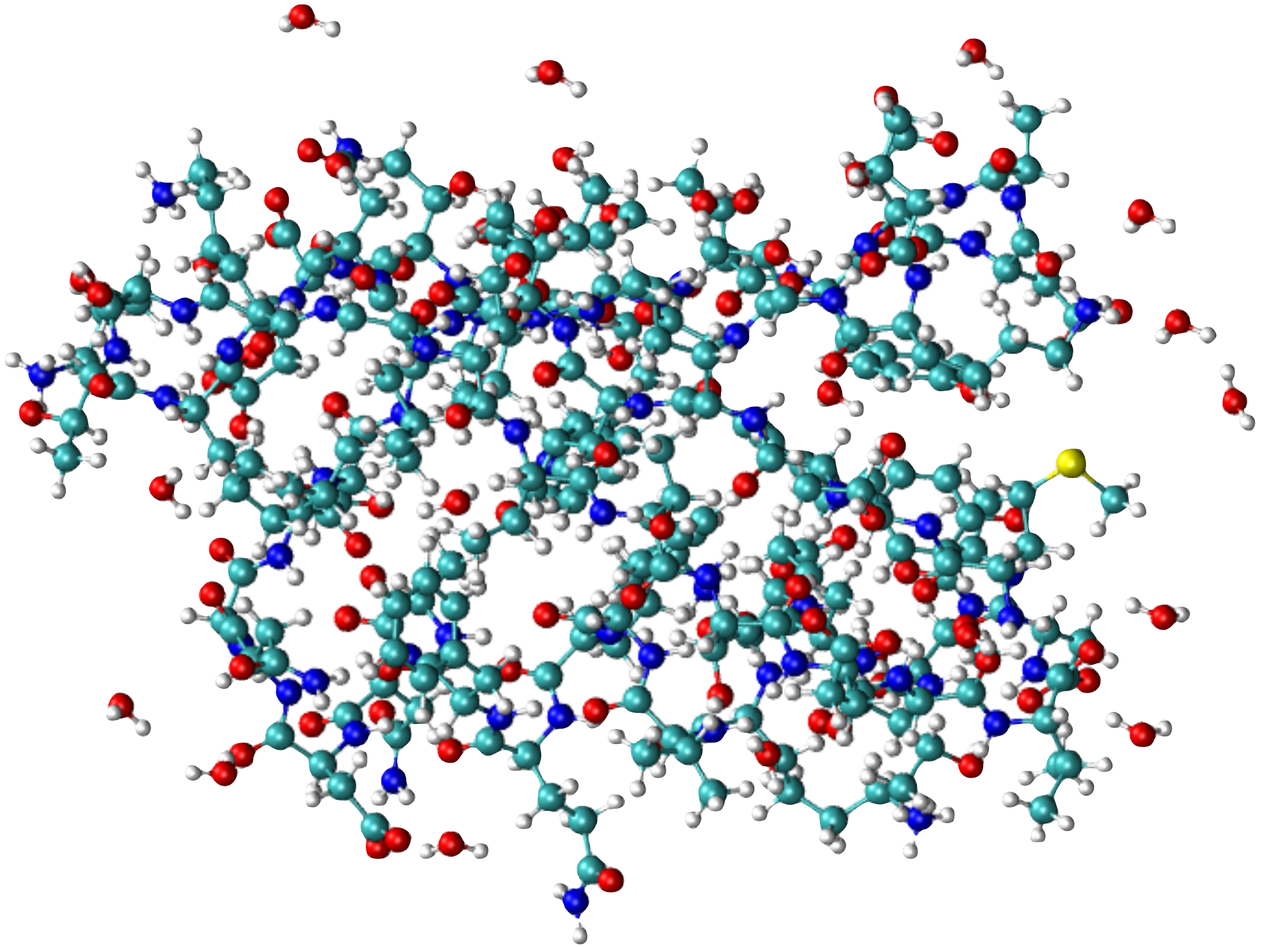
- **Space-fill, colored by evolutionary conservation (biologically important regions)**

The protein in the figure (triose phosphate isomerase, PDB entry 1amk) is shown in spacefill representation, with each residue colored by evolutionary conservation (turquoise - lowest, maroon - highest. See color-code in figure). The most conserved region is in the middle, where the natural substrate of the enzyme is bound.



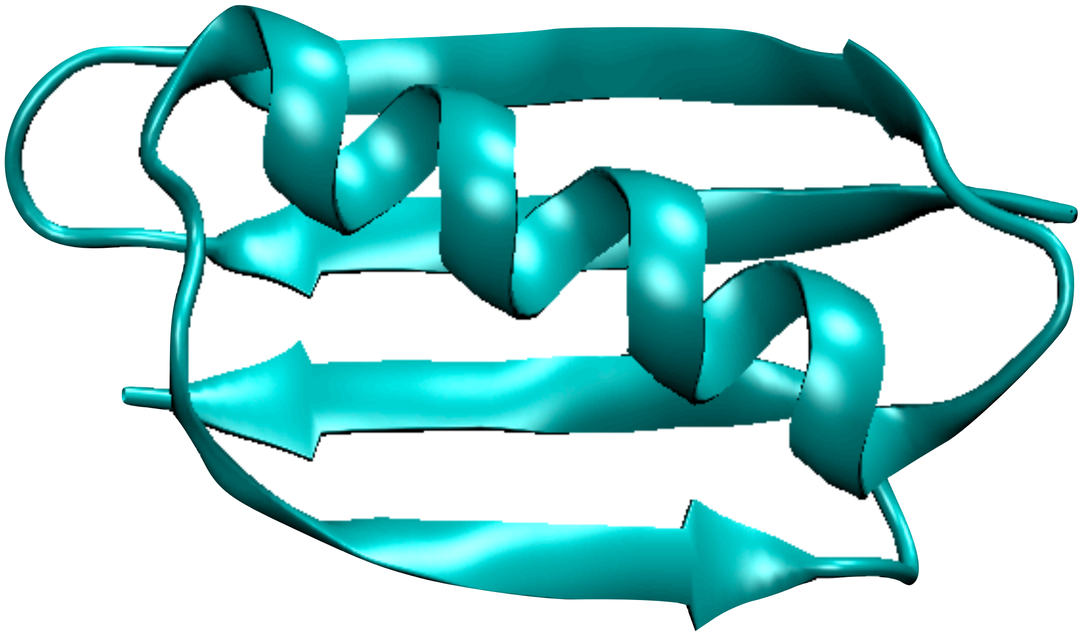
(http://consurf.tau.ac.il)

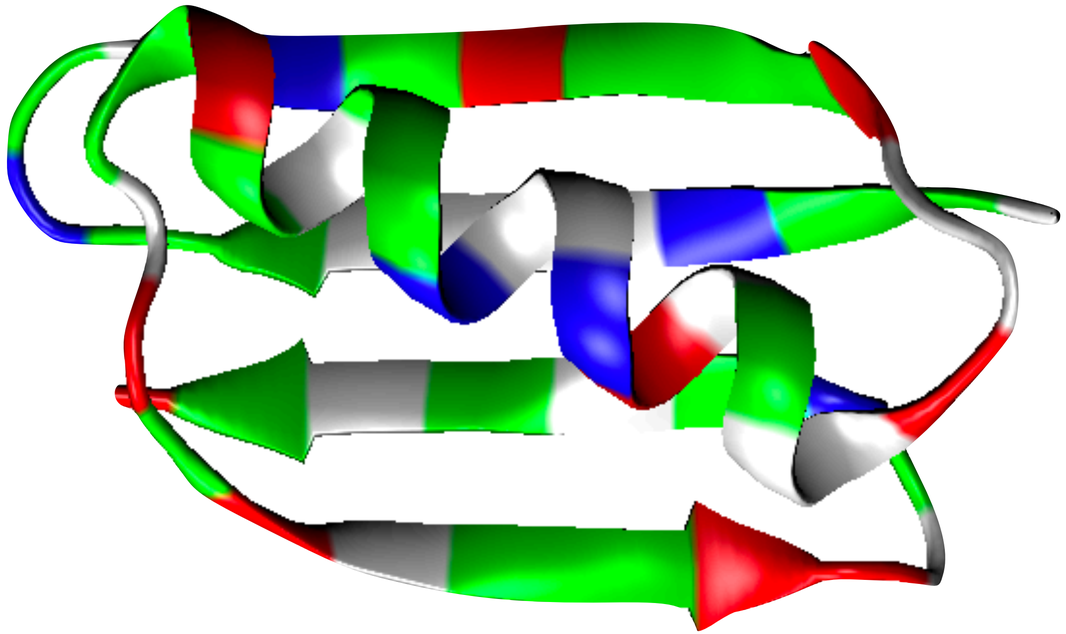
1PGB in Ball-and-Stick: showing all atoms determined



This is a 56 residues protein, determined by X-ray crystallography. In additions to all the protein atoms, also some trapped water are present.

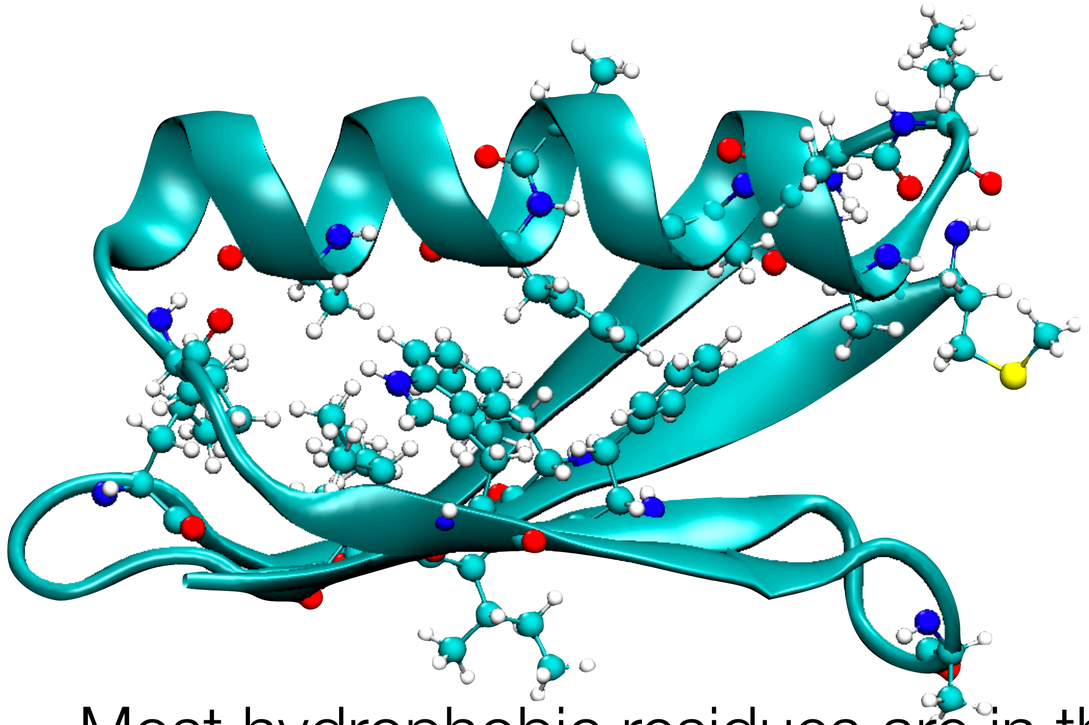
1PGB in cartoon: focusing on secondary structures

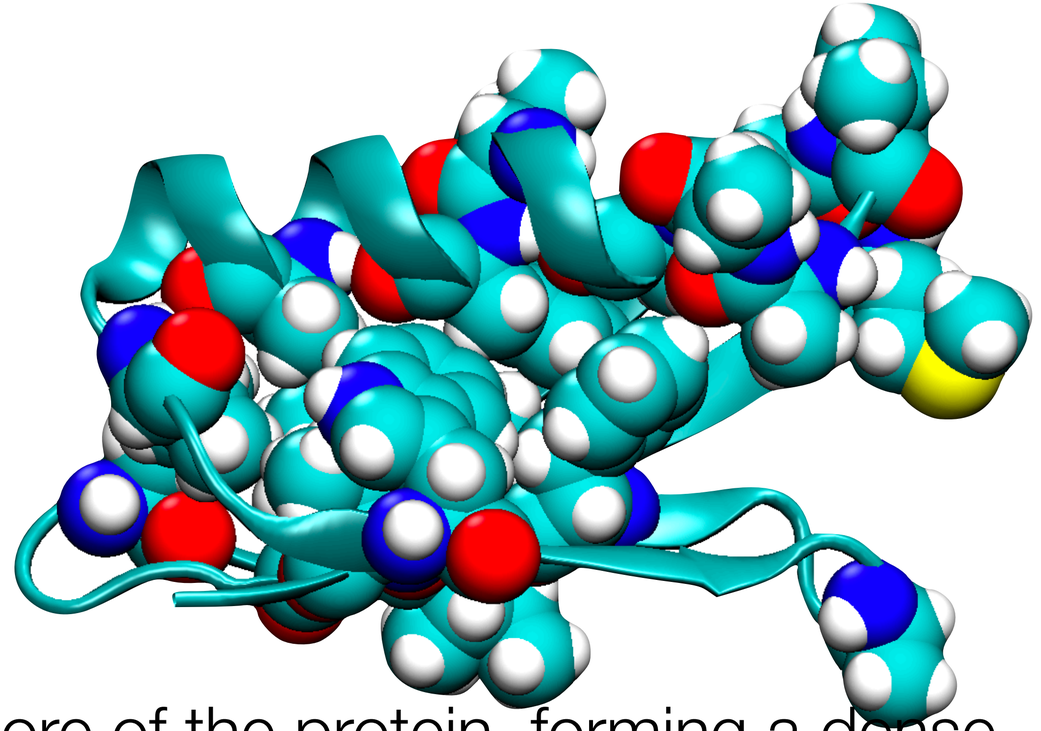




Coloring by residue type (polar: green/non-polar: white/ positive: blue/negative: red) highlights typical pattern of secondary structure amino acidic composition

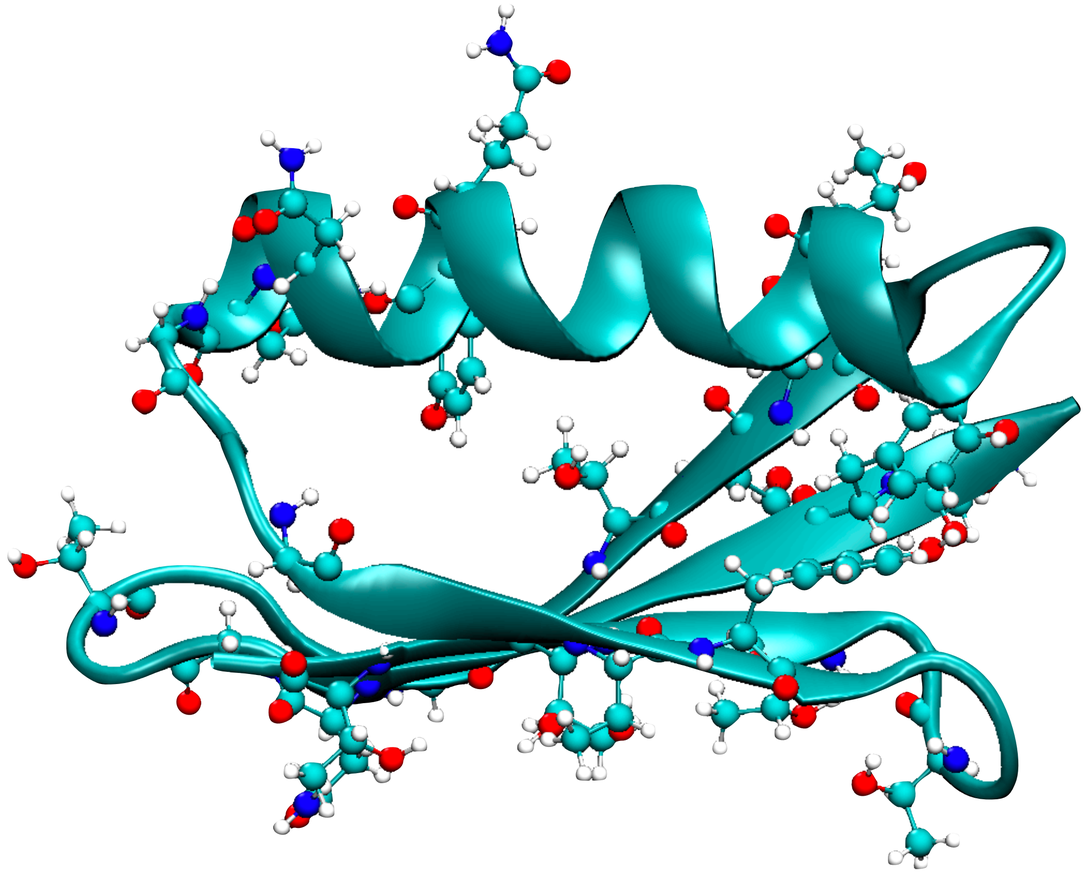
1PGB adding hydrophobic residues

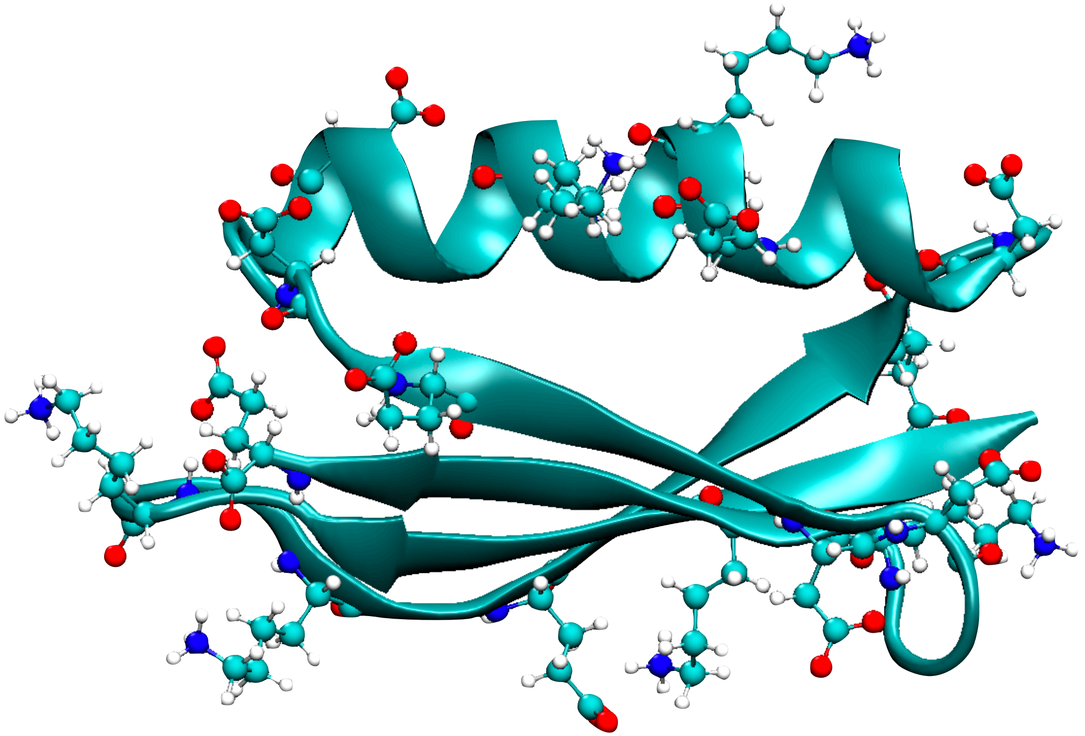




Most hydrophobic residues are in the core of the protein, forming a dense packing (looking using correct atomic radii), but some are also on the surface, possible hint for protein-protein interaction sites?

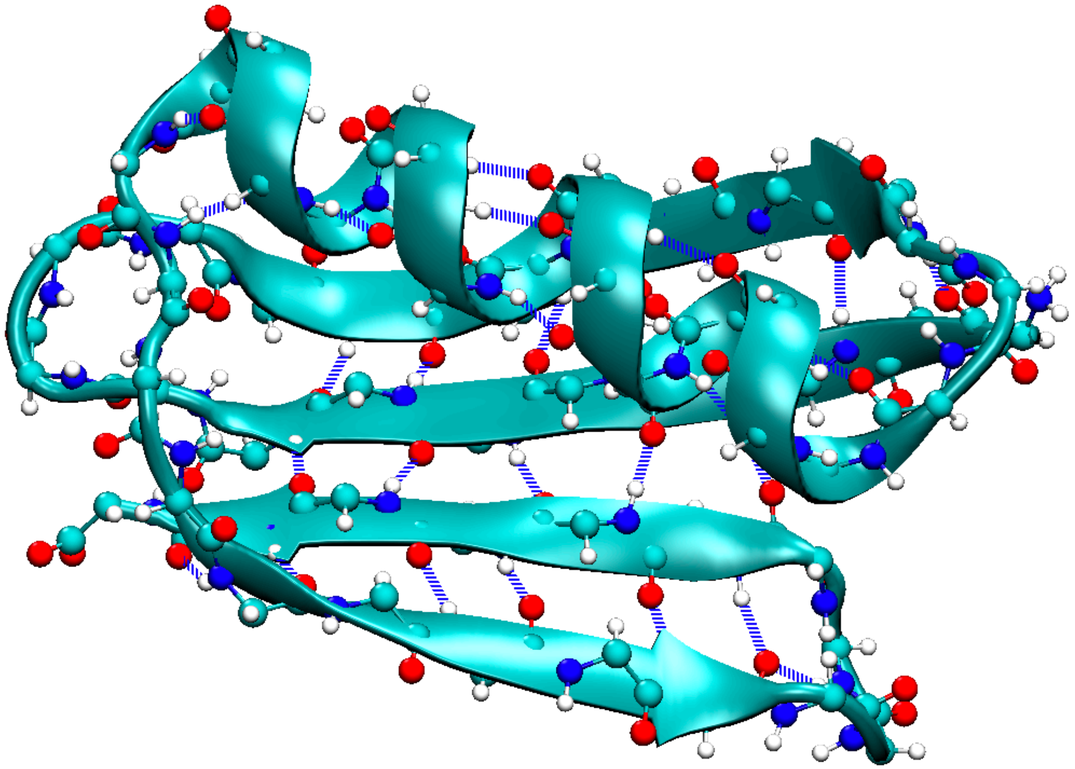
1PGB showing only Polar and Charged residues



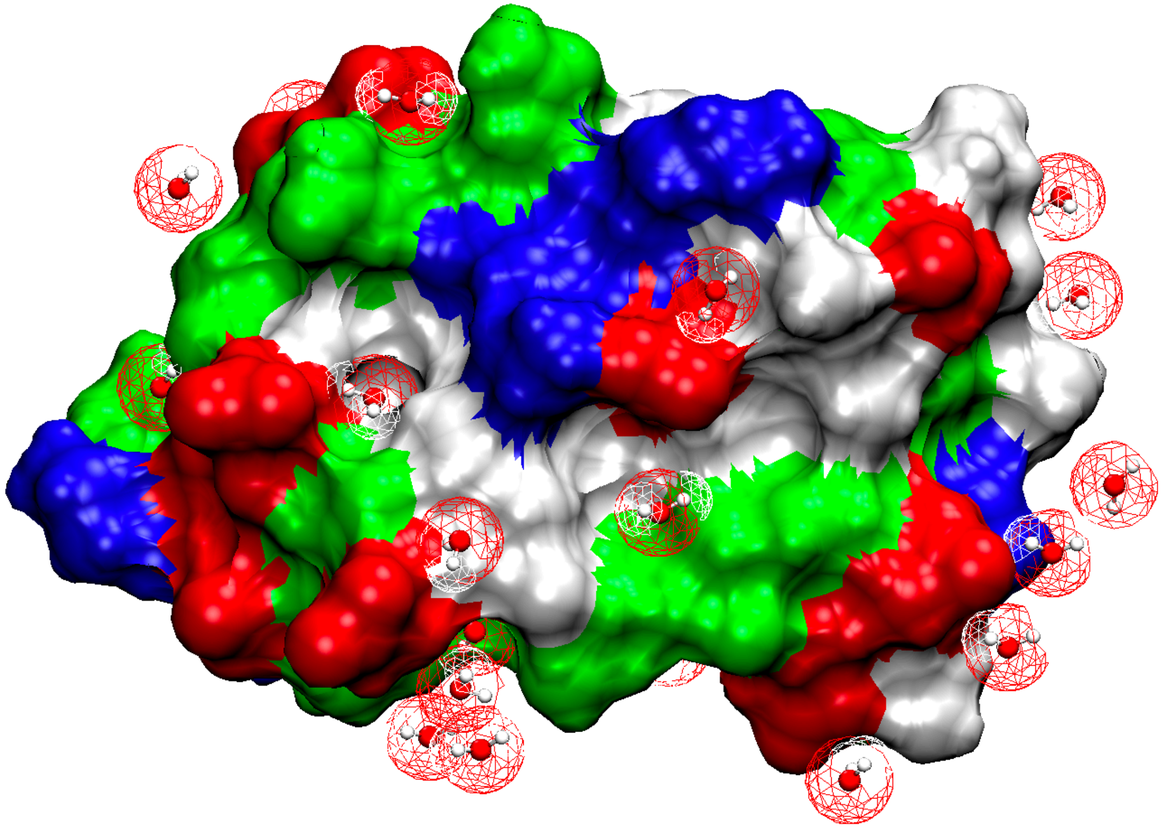


Polar and charged aminoacid are mostly on the surface and in the loops

1PGB: looking at hydrogen bonds and solvent accessible surface area



Hydrogen bonds are usually defined geometrically
distance between donor and acceptor < 3.5
Angle between donor, acceptor and hydrogen < 30 deg



SASA is coloured by residue type highlighting polar and hydrophobic regions

Visualisation: Software

VMD: most used for molecular dynamics simulations

PyMol: high-quality graphics, scriptable in python

ChimeraX: high-quality graphics, very good for cryo-EM

Coot: focused on structure determination for X-Ray and Cryo-EM

Molecular Nodes: a Blender adds-on for 3D modelling and animation

NGLView: to be programmed in python