UNIVERSITY OF JOHANNESBURG

FACULTY	: Science
DEPARTMENT	: Biochemistry
CAMPUS	: APK
MODULE	: BIC8X02 PROTEIN BIOCHEMISTRY
SEMESTER	: First
EXAM	: **APS**

DATE : 17 JULY 2020 **SESSION**: 09:00-12:00

ASSESSOR : PROF AP KAPPO

MODERATOR : DR T MULAUDZI-MASUKU

DURATION : 3 HOURS **MARKS**: 100

INSTRUCTION : ANSWER ALL QUESTIONS

QUESTION 1:

(a) The diagram below is a circular dichroism spectroscopic measurement of RING finger domain. What information can you as a structural biologist extrapolate from the Figure 1? **10 Marks**

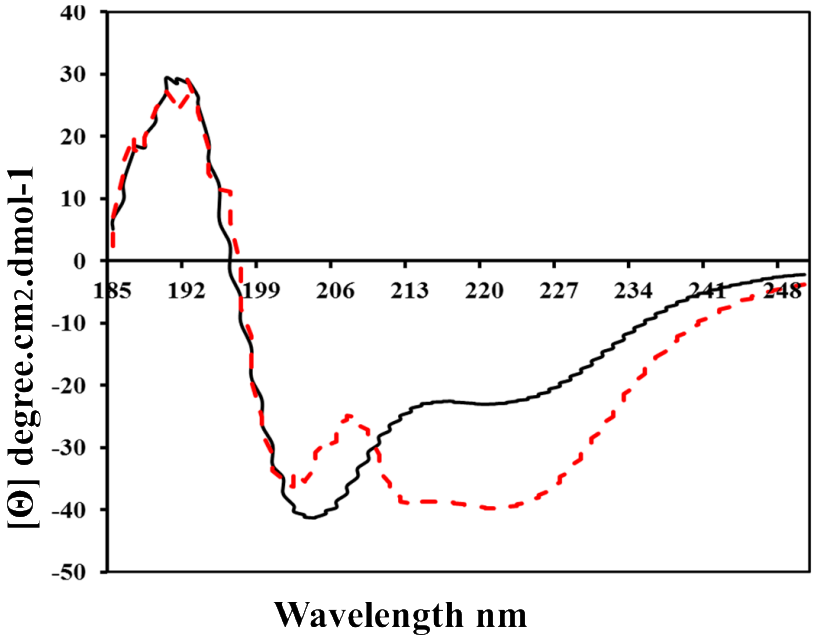
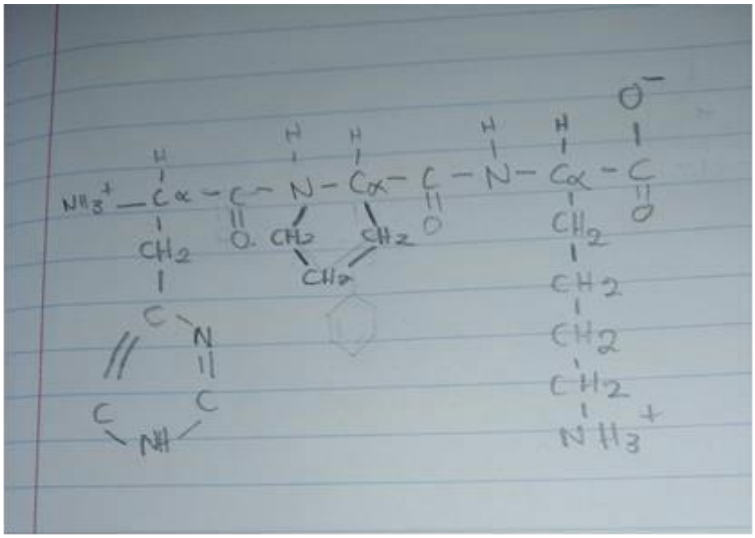


Figure 1: Circular dichroism spectra of a native (without 8M Urea: in black line) **and denatured** (with 8M Urea: in red dotted line) **RING finger protein.**

Before denaturation with 8M urea, the spectrum shows that the protein under study consist predominantly of helices, this is shown by the peak at approximately 190nm and two troughs between 200-210 and 220-230, respectively. Denaturation by urea then resulted in a decrease in the ellipticity this is because the hydrogen bonding is disrupted resulting in a decrease in the helical content of the protein and the trough starting from 213nm indicates that the predominance of β-sheets. So there is more disruption of helices which masked the presence of sheets which are now picked up following denaturation.

(b) Draw the chemical structure of the tripeptide: **H-P-K** (assuming the peptide preceding Pro is in the *cis* configuration) and indicate how many angles of internal rotation are there in the amino acid residue **K**. **5 Marks**

K (lysine) contains 5 internal (side chain) angles of rotation.



(c) Structural conformation of a protein can be measured by probing the fluorescence emitted and its recovery using a spectroscopic method called FRAP. Give a detailed description of the mechanism involved using the diagram below. **10 Marks**

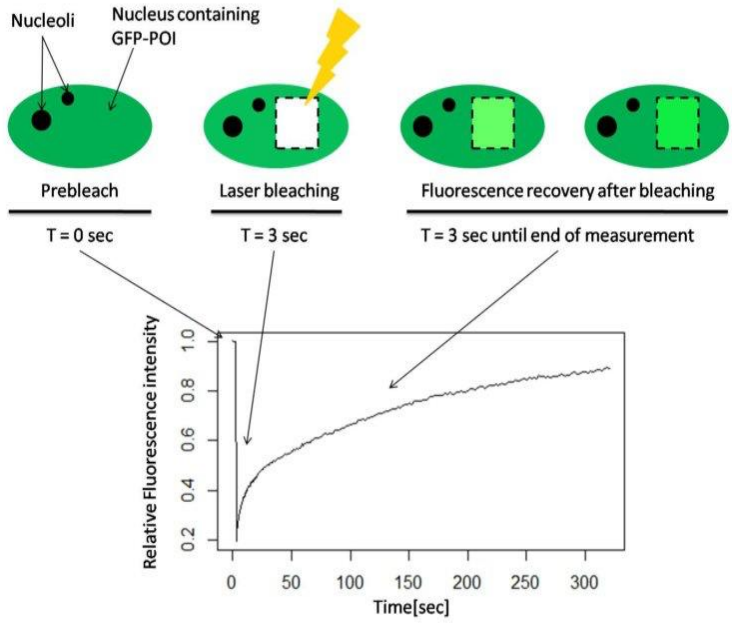


Figure 2: FRAP (Fluorescence Recovery After Photobleaching)

Fluorescence recovery after photobleaching (FRAP) is a fluorescence microscopy method that is utilized to study the mobility of fluorescently-labelled molecules in cells and allows for cellular localization. It also plays a role in molecular diffusion analysis, protein binding as well as biomolecule fluidity analyses. The molecules to be analysed need to first by tagged to a fluorescent dye and the initial fluorescence in the region of interest/study in the cell is measured, this is referred to as the pre-bleach phase (T= 0 sec- before a laser beam is focused onto the area) at which the relative fluorescence intensity is at its highest. Next, a laser beam of high intensity is then focussed on the area of interest, that is, the fluorescent molecules in this area are photobleached (this is a photochemical reaction), thus resulting in a permanent and

irreversible damage and thus the molecules lose their fluorescent tags and are rendered non- fluorescent and their mobility also ceases. So, no fluorescence will be observed in the photobleached area and the relative fluorescence intensity will be at its lowest (as in figure), this phase, at T= 3 seconds is referred to as the laser bleach phase. After some time, there will be lateral diffusion of other fluorescent molecules into the photobleached area thus resulting in a gradual increase or recovery of fluorescence in the area (this is indicated by the gradual increase in fluorescence intensity observed in the figure after T=3sec). It is this recovery in fluorescence which is measured overtime and gives information on the diffusion as well as mobility of molecules. Other examples of photochemical reactions include vision and photosynthesis

QUESTION 2:

(a) The heat shock family of proteins such as Hsp40, Hsp60, Hsp70 and Hsp90 play critical roles in the protein turnover machinery. However, their activity in some instances may not be optimal and thus some misfolded proteins, as well as some ageing proteins are shunted into cells where they experience a different fate. Explain this very important biochemical phenomenon. **10 Marks**

The misfolded proteins which could not be corrected or refolded by the intrinsic chaperonin activity are marked for degradation by a polyubiquitin chain, this process is referred to as protein turnover. Protein turnover refers to the controlled degradation of polyubiquitin-tagged proteins and it is coordinated by the 26S proteasome. The first part is ubiquitination during which the protein is tagged with ubiquitin molecules (76 amino acid long protein) in a process of three stapes catalysed by three enzymes. Firstly, the ubiquitin molecule is activated by means of ATP hydrolysis, that is, the E1 (ubiquitin-activating enzyme) harvests or makes use of the energy released from ATP hydrolysis (which results in AMP + PPi) to activate the ubiquitin molecule. The activated ubiquitin molecule is then attached to the active site of the E1 enzyme via a thioester bond (*i.e* binding to the sulfhydryl group of the E1 cysteine residue). Secondly, the activated ubiquitin molecule is transferred from E1 to E2 (ubiquitin-conjugating enzyme) to which it is also bound via a thioester bond. Then the conjugated EV enzyme to the activated ubiquitin is conjugated to the E3 (ubiquitin ligase) enzyme which is in turn bound to the substrate (*i.e* a damaged protein, misfolded protein which could not be refolded by chaperonin activity or a protein that has reached its half-life- as determined by the N- terminal residues). At this point, the ubiquitin molecule from the E2 bind to the epsilon amino group of the lysine 48 residue of the bound substrate by an isopeptide bond, and the substrate is released. The bound ubiquitin molecule is then joined by three more ubiquitin molecules, forming a polyubiquitin chain.

Once the polyubiquitin chain has been attached, the protein is then targeted for protein degradation by the 26S proteasome, which is an ATP-driven multi-subunit complex composed of two regulatory 19S subunits (which serve as caps) and a 20S

catalytic barrel which digests the protein. The 19S subunit recognizes and attached to the polyubiquitinated protein, once bound, the subunit will now use ATPase activity to unfold the protein such that it fits in the core. The 20S core contains an isopeptidase which cleaves off the polyubiquitin chain which is recycled and it then digests the protein into small 8-10 amino acid long peptides which cannot aggregate and are released. These peptides are then digested by cellular proteases into individual amino acids which are re-used for other cellular roles.

(b) Describe the process of determining the structure a recombinant 40kDa Arginase enzyme, which have been cloned into a pGEX 6P-2 vector using macromolecular X-ray crystallography. **15 marks**

The structure of the 15kDa Arginase enzyme can be determines by first over-expressing the enzyme protein in bacteria before solving the structure using X-ray crystallography. The expressed enzyme needs to be extracted and purified. An *E. coli* host with cloning vectors that are inducible with IPTG or a carbon source. T7 RNA polymerase promoter are used to express proteins which are present in the *E. coli* host. The vector used here is a pET vector containing a 6XHis tag. The solubility of proteins and enzymes can be improved as well as the ease of purification of the protein can be done using a protein tag such as the 6XHis affinity tag present in the vector. Tags arises from protein vectors in the expression system and are expressed together with the protein to form a fusion protein. The tags contribute towards the solubility of the protein where tags can get folded into the protein making them more soluble. His tags are less than 1kDa and are significantly small enough compared to the 15kDa enzyme to get folded into the 3D conformation. Affinity chromatography is used to purify tagged proteins using a column with an affinity to the tag; as the non-specific proteins will be eluted and the tagged proteins will bind to the affinity column. His-tagged protein such as the Arginase enzyme can be purified using a Ni-NTA column. Nickel binds to the histidine in the tags due to its high affinity to the imidazole groups, the tagged and bound protein can be eluted using an elution buffer containing Tris and a high concentration of imidazole, which breaks the interaction between Nickel and His. Elution of pure Arginase enzyme occurs as the imidazole competes with the His and binds more strongly to the Nickel beads on the column. The eluted Arginase enzyme may not be homogenous and may still contain some impurities hence, a second round of affinity chromatography or even ion-exchange chromatography may be done.

As a final polishing purification step (removes impurities), size exclusion chromatography will be done to further purify the protein and it also allows for buffer exchange which means that the protein can be changed into the buffer with the pH of choice for structural determination. Once the protein is purified to homogeneity and it is soluble, X-ray crystallography will then be used, this method is advantageous in that it allows for the determination of protein complexes and proteins of higher masses and unlike NMR, it has a lot of software which makes the process of structural determination much easier (less tedious). The protein will then be mixed with various concentrations of the precipitant and crystallization will be done by either hanging drop or sitting drop, in both processes, vapour diffusion is used during which the water vapour from the protein solution drop will drop into the precipitant in the reservoir until a state of equilibrium is reached- where the osmolarity of the protein

and that in the reservoir is equal, the dehydration results in a state of supersaturation (water is removed from the protein solution while avoiding aggregation), precipitation occurs followed by nucleation. The supersaturated state is important for crystallization, once the nuclear crystals are formed and grow into well-ordered crystals (a requirement of X-ray crystallography), they are then sent to a synchrotron where they will be bombarded with a beam of X-rays which diffracts the atoms in the structure giving rise to the diffraction pattern. The rays will diffract at a distance depending or proportional to that of the atoms. The diffraction pattern will be phased/ processed by measuring the intensities and atomic distances giving rise to the electron density map which undergoes fitting and ultimately gives the atomic model or cartoon structure. Refinement may be done, which involves the manual addition of the missing portions from the diffraction pattern.

QUESTION 3:

(a) The distance dependence of the van der Waals interaction between two non-bonded carbon atoms as shown in the **Figure 3** is a classical example of stabilization of protein native state. Describe this phenomenon in detail. **10 marks**

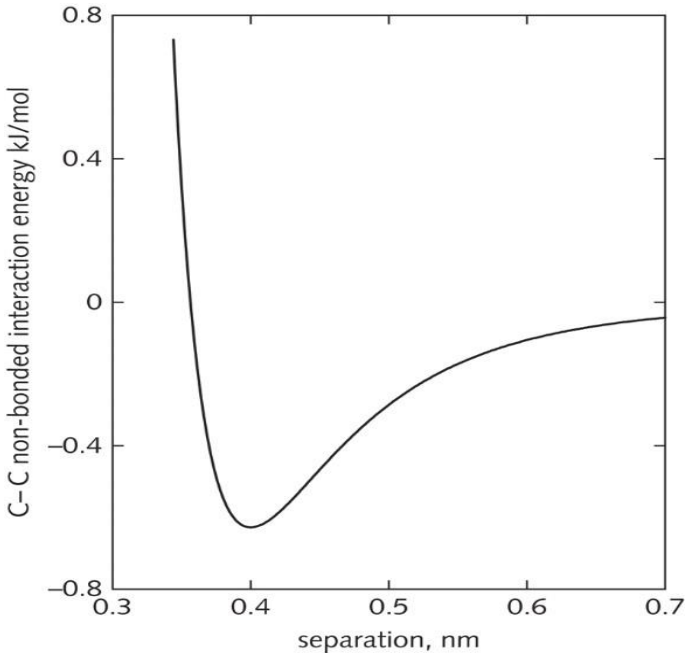
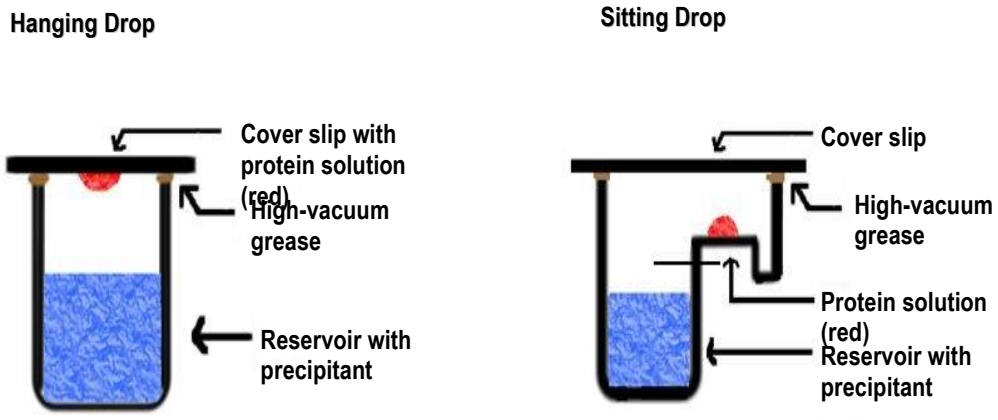


Figure 3: Van der Waals interaction between 2 non-bonded carbon atoms.

For stabilisation of the protein by van der Waals' forces to occur the non-bonded C-C must be in close proximity. From the figure, the maximum c-c non-bonded interaction energy is shown at 8 KJ/mol and as the interaction energy drops/decreases there is a smaller separation distance observed between the C-C carbons of the side chains. The lowest interaction energy is seen at 0.4 nm separation distance, this is the preferred state- that is, to be energetically favourable for protein stabilisation, the C-C non-

bonded interaction energy has to be small and this can only be obtained at a smaller separation distance when the atoms are closer together. That is, the smaller the separation distance, the closer the C-C atoms are in space, this then means that there is a stronger van der Waals' (repulsive force) and the stronger this force is, the more stabilised the native state of the protein will be. Conversely, when the interaction energy increases again (*i.e* from approximately -0.65 or -0.6 to a more positive value), there is a corresponding increase in the separation distance (as observed by the gradual increase in the graph/curve, which means that the higher the C-C non- bonded interaction energy, the wider the separation or the more distant the carbons of the side chains will be from each other and thus the weaker the van der Waals' forces will be. So, the van der Waals' forces are the weakest at 0.7nm separation distance as opposed to at 0.4 nm. Furthermore, as the side chains are stabilized, the non-bonded atoms are pushed together towards the core or interior of the protein, this forms a cavity in the interior. As more and more side chains interact to fill up this cavity, there is a resultant dense packing in the protein interior and this further stabilizes the native state.

(b) Protein crystals can be produced using either hanging drop or sitting drop methods (**Figure 4).** Describe the two processes using the diagram below. **15 marks**



Refer to Protein structure determination slides (especially slide 15)

-This is certainly the most common and cost-effective way of producing crystals

-A drop is made by mixing protein with various concentrations of precipitant solution

-This is either suspended under a silicone-coated cover slip (hanging drop) or placed on an elevated post (sitting drop) above a liquid reservoir

-Water diffuses from the drop through air-gap separation into the reservoir until equilibrium is reached

-The drop now becomes dehydrated and super-saturated, causing crystals to form

QUESTION 4:

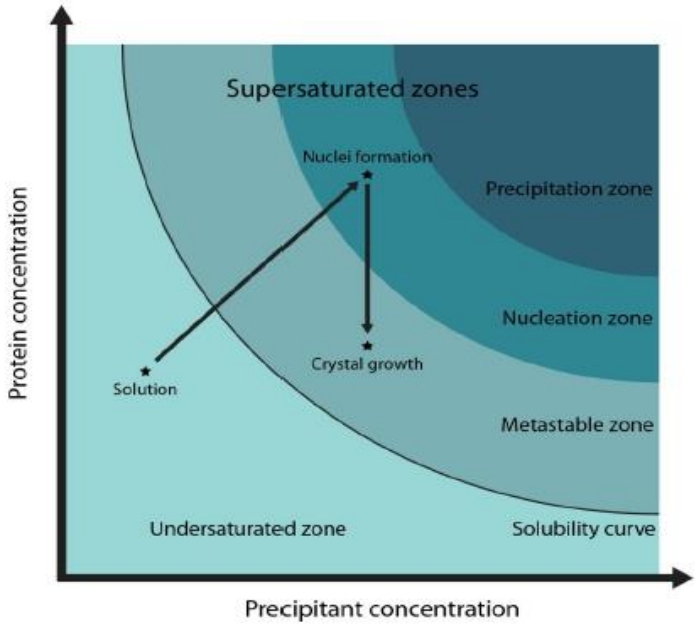


Figure 4: Process of crystallization: the phase diagram

(a) Figure 4 is a phase diagram describing the process of crystallization. Explain the various stages of this process using the diagram. **15 Marks**

The diagram above shows the crystallization process which can be summed up in three processes namely, nucleation, growth and termination of growth seen in the different zones or phases above. Firstly, an unsaturated solution is subjected to a state of supersaturation, this can be done through the process of vapour diffusion during which the water vapour from the protein solution in either sitting or hanging drop drops into the precipitant in the reservoir until a state of osmolarity equilibrium is reached. The dehydration results in a state of supersaturation (water is removed from the protein solution while avoiding aggregation), precipitation occurs and from the precipitation zone, then comes the nucleation zone which is the zone/phase during which crystals start forming as small nuclear crystals, these undergo growth (crystal growth) over time until they reach a stage where big, well-ordered crystals are formed and observed in the metastable zone. It is these crystals which are then sent to the synchroton (once it has been confirmed that they are protein and not salt crystals).

(b) NMR spectroscopy and macromolecular X-ray crystallography are some of the methods that can be used in determining the molecular structure of proteins. Compare and contrast these two methods. **10 Marks**

NMR spectroscopy:
a. is dependent on the size of the magnet in the spectrometer

b. can only solve structure of small sized proteins (~30 kDa) using an 800-900 MHz magnet.
c. An advantage of NMR is that it allows you to solve protein structure at the physiological pH (6-7)
d. When solving protein structure using NMR, always use a sodium phosphate buffer (between pH 6.2 and 6.5) – that is to do gel filtration using sodium phosphate buffer as well.

X-ray crystallography:
a. Can solve protein structure of larger size (120-150 kDa) but the bottleneck of this requires protein crystals that perfectly diffract light.
b. How are the crystals formed?
Compress the protein into a small space (constrict the protein solution in a small space), therefore protein loses water and precipitates, then it becomes nucleated and subsequently forms crystals. The protein structure is solved with the protein crystals in a model liquid.