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PRINCIPLES OF PROTEIN CRYSTALLIZATION

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ABSTRACT

This study discussed about the principles of protein crystallization. Since the first established remark of protein crystallization about 160 years back the growth protein crystal has enhanced into a comprehensive field of research with several applications. In this an overview of protein crystallization, their principles with some essential aspects of protein crystallization is described. Protein crystallization constitutes a comprehensive research field rather varied from conventional crystallization of small molecule or in-organic compounds. The most similarly used techniques of protein crystallization are explained together with their major principles and the approaches used to detect applicable conditions of crystallization of a specific protein.

Index Terms— Protein Crystallization, Principles of Protein crystallization, Protein Crystallization techniques.

I. INTRODUCTION TO PROTEIN CRYSTALLIZATION

According to McPherson A [1] there are presently two major processes for determining the 3 dimensional protein structure. They are: X-ray crystallography and NMR (Nuclear Magnetic Resonance) Spectroscopy. The Nuclear Magnetic Resonance Spectroscopy process is able to solve the protein's atomic structure with an upper limit molecular weight of approximately 25000 Da (220 amino acids i.e. 25 kDa), whereas the X-ray process is more applicable for resolving macromolecular complexes or larger protein structure. On the X-ray crystal structures the developmental studies of hemoglobin (1955) and myoglobin (1950) were appreciated in 1962 with the Nobel award in Chemistry. This identification not only emphasized the significance of X-ray crystallography but also transmitted the rise of structural biology as an important field in life sciences. In the X-ray crystal structure determination the first step which is always the most critical step, is the development of adequately big crystals of protein. A crystal is a 3 dimensional periodic building block arrangement and these building blocks are considered as the molecules of proteins.

[3] have mentioned that the protein molecules solubility must be reduced in order to reach at a crystalline form beginning of a dissolved form. As an universal rule, lowering down the protein solution solubility will outcome in amorphous protein precipitate formation. However if proper conditions are chosen so that additional patches are existing on adjacent protein molecule surfaces in solution, particular attractive interactions can exist between the molecules of protein. This can lead to protein crystallization if these interactions are favorable geometrically.

Hahn [4] has mentioned that the crystallization process can be distinguished into 2 steps: 1) crystal growth; and 2) the nucleation method. With properly chosen conditions, crystal growth and nucleation can exist within the phase diagram's supersaturated regions as shown in the below figure:

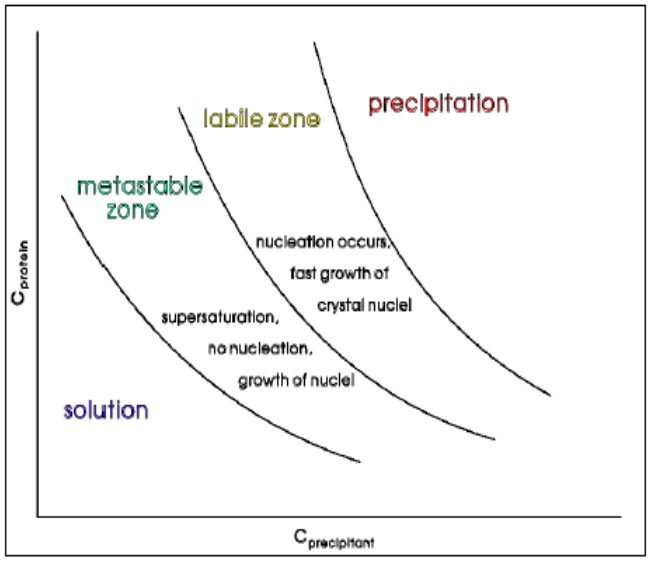

Figure 1: Protein precipitant mixture schematic phase diagram

As can be represented from the above figure 1 the crystallization needs the supersaturated protein precipitant solution formation, that is a solution which consists of numerous protein molecules than would melt under normal circumstances. Unluckily one requires a bigger supersaturation degree for nucleation as for development and therefore various crystal growth and nucleation processes are always critical to manage individually. The range of nucleation is also referred as the labile zone, while the range of growth is referred as the metastable zone. Bergfors [5] has described that the researcher must pull the solution of protein into the labile zone for crystal nucleation to exist, which is also a fast growing region of crystal nuclei existence. Here in lies the hazard keeping the solution of protein within the labile zone for a more time period will outcome in fast development of numerous crystal nuclei and generates a surplus of little crystals. Since one is concerned in acquiring big crystals of protein i.e. 0.5 millimeter in length, it is essential that not numerous nuclei are constituted. This refers that for a successful procedure the mixture of precipitant and protein must approach the zone of nucleation slowly so that the emerging nuclei have much time to develop.

Hunt [6] has mentioned that from a stable solution to a supersaturated solution the transition can be gained by regulating the position of protein-precipitant mixture in the above figure. This can be gained by developing either the precipitant or protein concentration either in the horizontal or vertical axes as shown in the above figure 1. The physical method of affecting a modification in concentration can be undertaken through diffusion or dialysis method. Both are described by the material transportation. For protein crystallization the most appropriate method is a vapor diffusion method.

Iwata [7] has described that however, the protein crystallization is not only a superior component to gain information about the 3 dimensional structure of protein but also a very concerning concept for studies of crystal growth in its own law. A contrast to 'conventional', little molecule (always salts) of crystal growth systems, proteins shows similarly a gradual kinetics of growth and have units of growth with a big size because single molecules of protein are very big. These facts permits the researcher to more study the mechanisms of the crystal growth par instance by means of microscopy observations of *in situ* atomic force. Therefore, it can be inferred that protein crystallization is considered as the best system model for normal crystallization from solution.

II. HISTORY OF PROTEIN CRYSTALLIZATION

The protein crystal growth history begun around 160 years back. Keppler recommended in 1611 that the snowflake crystals hexagonal symmetry were derived from a continuous arrangement of tiny units just like a brick which gave him the idea of a crystal. In 1840 the first protein crystallization observation was established by Hünefeld. The hemoglobin protein from earthworm, was acquired as flat plate-like crystals when the blood of a worm was squeezed between the 2 glass slides and permitted to dry

gradually. This remark stated clearly that the crystals of protein can be generated by managing evaporation of concentrated solution of protein i.e. protein crystals can be generated by gradual dehydration. This is the foundation for several technologies which was used nowadays [8].

Waller et al. [10] has mentioned that the first examination that took up protein crystallization, concentrated on hemoglobin (of numerous species of animal) for next 15 years. Unluckily all the crystals of blood existed to have developed more or less fortunate through 1850 and no researcher had recommended general methods for their directed growth. Funke was the first person to invent reproducible and successful processes for hemoglobin crystal growth who established a set of articles on the essential hemoglobin growth of crystals. After hemoglobin, in the period from 1850 to 1990 the next class of proteins to be examined were the reserve proteins of plant seed mainly the so referred 'globulins'. The methods that were enhanced to crystallize these proteins consists of protein extraction into salt solutions followed by gradual cooling, dialysis of seed extract salt solution thoroughly against protein solutions treatment and distilled water with ether, alcohol or acetone. In these processes the researcher detects the exploitation of numerous approaches now in similar use:

Similarly PeirreWery and Schevitz [11] have described that differences in temperature under stable conditions of solution, dialysis against the solutions of less Ionic strength (to take benefit of less solubility of several proteins at concentrations of less ambient salt) and the organic solvent usage as crystallizing or 'precipitating' agents'. Similarly Nanev and Tsekova[12] has described that in the same period the plant seed proteins work was undertaken common efforts were performed to crystallize the horse serum and hen egg albumin proteins. For their crystallization the methods used numerous recommendations of Hofmeister considering the proteins salting out' by greater salt ion concentrations and the protein precipitation by careful adjustment of pH value In 1925 the first enzyme urease was crystallized by Sumner and at the same time insulin the first hormone was too crystallized. Difficult to crystallization of insulin was the addition of divalent ions of zinc. This was one of the first instances of crystal growth raised by the addition of metal ions.

According to Chernov and Komatsu [13] the basis for crystallizing proteins, until 1930s especially enzymes, was to provide a technology for purifying a particular protein from a complicate extract, or to describe the purity of preparation. However specific diffractionists of X-ray crystallography in the late 1930s started to turn their attention to crystals of protein as a basis of structural information about macromolecules of biology for reasons referred in former cases. This concern is undertaken till now. The concern of X-ray diffractionists was impacted in raising efforts to develop high quality protein crystals reproducibly but also led to efforts to higher the rates of success and to develop the process of crystallization. For the latter the background is constituted by the fact that with remarkable advancements in computing and data gathering techniques and with biotechnology and pharmacology revolution the task for new macro-molecular crystals surpassed their supply greatly.

Robert et al.[14] have described that presently the bottleneck in resolving the issue of reduced crystallization yields is constituted mainly by lack of insight. Numerous approaches of protein crystallization are based on the principle of trial-and-error while insight in major methods at work is prohibited. As a result still several proteins withstand crystallization for unknown causes. Presently the challenge is to acquire enough knowledge about the methods at work and, through this knowledge, to enhance more directed processes to be able to crystallize any protein readily.

III. PRINCIPLES OF PROTEIN CRYSTALLIZATION

According to McPherson et al., [16] in the X-ray structural protein analysis acquiring appropriate single crystals is the minimum understood step. The protein crystallization science is an undeveloped area, although concern is developing spurred

particularly by the experiments of microgravity in space flights. Mainly protein crystallization is a trial-and-error method in which the protein is precipitated gradually from its solution. In this process the crystallization nuclei, impurities presence and other unknown factors plays an essential role. As a general norm however, the purer the protein, better will be the opportunities to develop crystals. The protein crystallographer purity needs are more stringent and varied than the biochemist needs who would be fulfilled if for instance the enzyme's catalytic activity is highly adequate. On the other side Moreno et al. [17] in order to gain protein crystallization not only other compounds must be absent, but entire molecules of protein must have similar surface properties, particularly similar charge distribution on their surface, as this impacts the molecule packing in the crystal. In protein crystallization mass spectrometry is a valuable component that is in verifying the recombinant protein expression, protein constructs nature, derivatives of heavy atom and the purity of preparation [18]. The protein crystallization includes 4 important steps. They are:

1. The protein purity is decided. If it is not pure extremely further purification will be essential normally to gain crystallization.

2. In a proper solvent the protein is dissolved from which it must be precipitated by an organic compound or salt. Normally the solvent is a solution of water–buffer sometimes with organic solvents such as MPD (2-methyl-2,4-pentanediol)
added. Generally the solution of precipitant is also added, but only to such concentration that the precipitate does not yet enhanced. The proteins of membranes which are insoluble in a water organic solvent or water–buffer needs a detergent.

3. Cherezov et al. [19] has mentioned that the solution is lead to supersaturation. Small aggregates are constituted in this step which are the crystal growth nuclei. For small molecule crystallization which is perceived much better than the protein crystallization, the rising structure of nuclei needs surface tension energy provision. The crystal growth starts once the energy barrier has been proceeded. The barrier of energy is simple to overcome at a greater supersaturation level. Therefore, spontaneous structure of nuclei is gained best at a greater supersaturation. The researchers consider that this is also real for the protein crystallization. Nuclei formation can be studied as a supersaturation function and other variables by numerous techniques, including electron microscopy, light scattering and depolarization fluorescence.

4. Contrary to that Baird, Scott and Kim [20] have pointed out that the actual crystal growth can start once nuclei have constituted. The new molecule attachment to the growing crystal surface exists at surface steps for less molecular weight compounds. This is because in such positions the binding energy is bigger than if the molecule joins to a flat surface. These steps are either made by crystalline order defects or exist at nuclei constituted randomly on the surface.

Thus the theory infers that better crystals develop by reducing supersaturation to a lesser level; maintaining a greater supersaturation would outcome in the formation of numerous nuclei and, therefore, several little crystals as shown in the below figure:

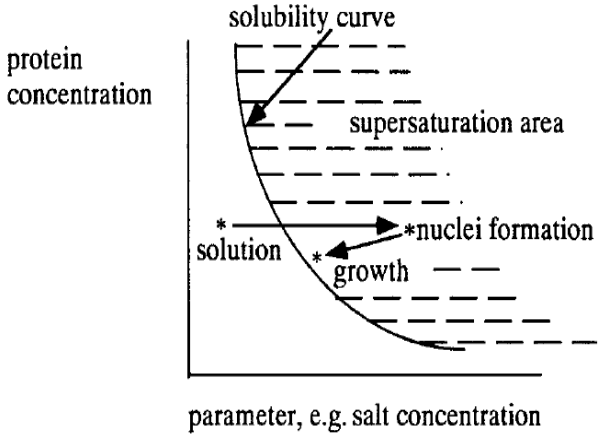

Figure 2: Protein solubility curve

According to Ries-Kautt and Ducruix [21] the crystals must develop gradually to attain a maximum extent of order in their structure. In practice, this major norm is not often respected. The simpler way to modify the super-naturation degree is by modifying precipitating gent concentration or the temperature. Protein precipitation can be gained by

increased number of salts, such as an organic solvent or PEG (polyethyleneglycol). A salt acts in two ways as a precipitating agent. On the protein molecule's surface the salt ions secure electric charges thereby reducing the disgutive forces between molecules.

Cherezov, Fersi and Caffrey [22] have described that in addition, a small amount of water is prevented and not available to protein because of the production of the hydration layer around the ions of salt. Ammonium sulfate is the most familiar salt. Its benefits are that it is not harmful and highly soluble to most proteins even in greater concentration. PEG (Polyethyleneglycol) has a greater water affinity and just as salt PEG can prevent water. Moreover, it causes solubility of protein in a varied manner. Similarly DaCosta and Baenziger [23] have described that Polyethyleneglycol molecules cannot approach the molecular surface of protein closer than PEG molecule radius and a layer that is unavailable for mid of PEG molecules encloses the molecules of protein. These layers overlap partly and the inaccessible region becomes less if the protein molecules aggregate. As an outcome the available region becomes bigger. For polyethyleneglycol molecules there is much space available, their entropy develops at the expense of some entropy of protein and the system's free energy reduces. This incident with aggregated protein is the most feasible in the polyethyleneglycol presence.

According to Garavito and Ferguson-Miller [24] for protein crystallization the most similar organic solvent is 2-methy-2, 4 pentanediol. Organic solvents often have an electrostatic effect. They reduce the dielectric medium constant. Around the protein molecules electric forces become powerful and this compresses the ions electric double layer. The protein molecules can aggregate in a feasible orientation and the molecules can approach each other closer. Some proteins are water soluble poorly but do melt if a little amount of salt (much littler than for salting out) is added. Raveli et al. [25] has described that the protein precipitates by eliminating the salt. This "salting in" effect is described as an outcome of a rivalry between ions in the solution and charged groups on the molecular surface of the protein. The protein molecules are not enclosed by an unique double layer in the absence of solvent ions and can integrate by Coulomb attraction between converse charges on various molecules of protein. A double layer constitutes around the protein molecules if a little number of ions are added. These layers do not repel and penetrate each other. Other processes to reduce the solubility of proteins are modifying the temperature or the solution's pH value.

Ravelli et al. [26] has described that to sum up the normal process for protein crystallization is as described below:

1. Carefully verify the purity.

2a.Gradually develop the precipitant concentration such as an organic solvent, salt or polyethyleneglycol; or

2b.Modify the temperature or pH value.

The amount of available protein for experiments of crystallization in practice is always less. To decide the best conditions of crystallization normally, it is essential to undertake a bigger number of procedures ; hence, a reduced number of protein must be used per procedure.The reasonable size of an individual protein crystal weighs (0.2×0.2×0.2mm=0.008mm3) approximately 10µgram. Michael [27] has mentioned that 1 milligram of purified protein is adequate to carry out around 100 crystallization procedures. The proteins of membrane are notoriously critical and insoluble in water to crystallize. The proteins conventional strategy is to use detergents to soluble them in aqueous solution and then pursue one of the methods for water-soluble proteins. Landau and Rosenbusch [28] implemented a new process that could be likely for membrane protein crystallization. They used the phases of the lipids cube as matrices of crystallization for different compounds. The phases of lipids cubes are liquid crystals with a symmetry of cubes. They can be constituted in a combination of water and lipids . Numerous kinds of lipid cube phases occurs. Landau et al. [29] won in developing a well arranged membrane crystals of bacteriorhodopsin protein in a particular type of lipids cube phase. It exists to be viewed whether this

process will be fortunate in the more membrane protein crystallization.

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