A CRYSTALLISATION STUDY OF THE CHANNEL-FORMING MEMBRANE PEPTIDE ANTIBIOTIC, GRAMICIDIN A

by

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I declare this thesis is less than 100,000 words in length and that all of the work contained within was performed by myself unless otherwise stated.

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Abstract

The purpose of the research was to provide a thorough description of the process of crystallisation for the membrane peptide, gramicidin A. This issue will be addressed in two ways. First, I will examine the current literature concerning the theory and process of crystallisation, including issues like solubility and heterogeneity. Having provided and in depth discussion of the crystallisation theory, it will then be specifically applied to the study of ion channel, gramicidin A. From this study, a new set of crystallisation conditions for gramicidin were found in the presence of the detergent. Gramicidin provides a unique case because it represents an interesting union of compounds, small molecules and membrane proteins. For this reason, techniques for both types of compounds will be invoked to study gramicidin's solubility and crystallisation.

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PART I:

CRYSTALLISATION

CHAPTER 1.

HISTORY

The regular faces and edges of a crystal reflect the internal array of ordered molecules. It is this internal order that allows a crystal to diffract x-rays in a regular recurring pattern. The crystal is made up of repeating sections known as asymmetric units. The asymmetric unit is the smallest area that can be translated in three dimensions to form the crystal lattice. Due to the recurrence of symmetry relations of the unit cell, it reflects the x-ray beam in a regular way and provides a diffraction pattern. Before crystallisation was used as a precursor for structural determinations, biochemists used crystallisation as an indication of the purity of their samples (Ducruix and Geige 1992). Although a crystal may look like a perfect single crystal, it is actually a composite of many different crystal lattices. As the crystal builds upon a nucleation site, slight defects begin to form. As layer builds upon layer, these slight irregularities compound one another leading to mosaicity in the crystal. Despite these defects, the crystal is still an incredibly well ordered system. In fact, early researchers used the internal order of crystal to examine the nature of x-rays. The crystal was employed as a three dimensional diffraction grating because the intermolecular spacing of atoms in a crystal is similar to that of x-ray radiation. Through such experiments, Friedrich and Knipping were able to prove that x-rays were wavelike in nature and on the order of 1 Å in size (Glusker and Trueblood 1985) After this discovery, Bragg was able to determine the structure of sodium chloride salt crystals using x-ray radiation

(Bragg 1912). Since then, x-ray diffraction has been used to solve the structures of organic compounds, metal complexes, viruses, and proteins. But the rate limiting step in many of these structural determination is often the growth of well-ordered, single, large crystals. For this reason, it is important to look into the process which governs the formation of crystals.

As one might expect, it seems difficult to coax a protein out of a stable solution into an incredibly well ordered three-dimensional array. Although some of the theory from the well studied area of small molecule crystallogenesis is applicable to proteins, macromolecular behaviour is in general much more difficult to predict (Ducruix and Geige 1992). Values like free energy in solution, bonding energies, and solubility constants are well documented for ionic compounds making the system easier to predictably manipulate. On the other hand, proteins are more complicated because they are large, asymmetric, chiral molecules, composed of enantiomeric amino acids (Stryer 1975). In addition, they have large irregular surfaces with a variety of charge distributions depending on factors such as solvent, salt, pH, and temperature (McPherson 1982). All these factors, make a rational experiment design for crystallisation of proteins very complicated.

Despite the complicated nature of the system, the simplest means of understanding it is to examine the thermodynamic factors that influence the free energy of the system. As one would imagine it is difficult to prepare a well-ordered molecular array over a space that is large compared to the size of the molecule. As with any phase change, one must examine the free energy of the system. Intuitively, it may seem unusual that molecules would willingly give

up degrees of freedom and decrease the entropy of the system to arrange themselves in a precise repeating solid state. As with any thermodynamic situation, we must consider the free energy of entire system. The solution is constantly driving towards a state of equilibrium. So although the individual protein molecules give up rotational and translational degrees of freedom, the formation of numerous, stable chemical bonds within the lattice outweighs the entropic loss and drives the formation of crystals. During this drive for energy minimisation, the attractive interactions, like charge, steric, hydrophobic, or hydrophilic are maximised while the repulsive interactions are minimised (McPherson 1982). Problems arise from the fact that there are a number of local energy minima. One such energy minimum is observed when the protein is in solution. If there are not enough solvent molecules to surround the protein molecules, the system will be unstable and drive towards another energy minimum. To encourage this equilibrium shift, the solution is brought to a state of supersaturation. The problem is that there are a number of solubility minima, some corresponding to amorphous precipitate while others lead to crystals. It is sometimes difficult to entice the solution to move towards the right one, especially if little is known about the solubility of the protein. Usually amorphous precipitate forms when aggregation of the protein occurs very quickly. If the energy well of precipitation is shallow enough, crystals may grow from amorphous precipitate, but in most cases avoidance of precipitation is usually the best. By slowing down the speed in which the solution reaches a point of supersaturation, it allows the proteins adequate time to orient themselves in a crystal array, as opposed to the random aggregation

that occurs with precipitation. In addition to the speed at which supersaturation is reached, many other factors such as pH, salt concentration, temperature and protein concentration can all influence the solubility minima (McPherson 1982). The idea is to try and manipulate these factors in such a way to create an environment that promotes favourable interactions between protein molecules. For this reason, in order to obtain crystal growth a solution must be brought slowly to a point of minimum solubility and achieve a state of limited supersaturation.

CHAPTER 2.

STAGES OF CRYSTALLIZATION

In general, crystallisation occurs in three stages: nucleation, growth, and cessation of growth. Nucleation is the point where ordered aggregates begin to form. The ideal situation would be to bring the solution to a limited degree of supersaturation, form a few nucleation sites and then at a lower degree of supersaturation continue to grow. These conditions can be very hard to find. Often there are too many nucleation sites leading to a shower of microcrystals. This depletes the protein concentration, not leaving enough to grow large crystals. Seeding of crystals can be used as a technique to supply nucleation sites to a solution. In this way, the solution can be brought to a lower degree of supersaturation than that required for the nucleation step and the multitude of protein that comes out of solution will grow onto the seeded nucleation sites. Since nucleation and growth occur at different levels of supersaturation, it is important to examine them separately. A saturated solution is defined as a solution that "contains an amount of solute such that there cannot be either growth or dissolution if crystals are added to it (Ducruix and Geige 1992)." This relationship is governed by the thermodynamic equation of equilibrium between two phases:

$$m_c = m_s = m_o + RT \ln \gamma c$$

Where m_c is the chemical potential for protein in crystal form, m_s is the chemical potential for protein in solution, m_o is the standard chemical potential, R is the gas constant and T is the temperature in degrees Kelvin, γ is

the activity coefficient, and c is the protein concentration. At equilibrium the chemical potential of protein in crystal form is equal to that of the solution, but at supersaturation the chemical potential of the solution exceeds the chemical potential of the crystal. To induce supersaturation experimentally, we can change the right side of the equation to increase m_s. For example, increasing the temperature, T, or protein concentration will directly influence the chemical potential of protein in solution. Similarly, by manipulating the electrolyte concentration or the pressure, one can change the values of the activity coefficient and standard potential respectively. These types of parameters influence the equilibrium indirectly.

At the point of supersaturation, the concentration of protein is defined as c_s, or solubility. Although the official definition of supersaturation is the difference between the chemical potential of solution and the chemical potential of the crystal, a common approximation is usually the ratio of the difference between the protein concentration and the solubility over the solubility.

$$\frac{(c - c_s)}{c_s} \approx m_s - m_c$$

Although supersaturation is a requirement for nucleation, it must be noted that there are different degrees of supersaturation.

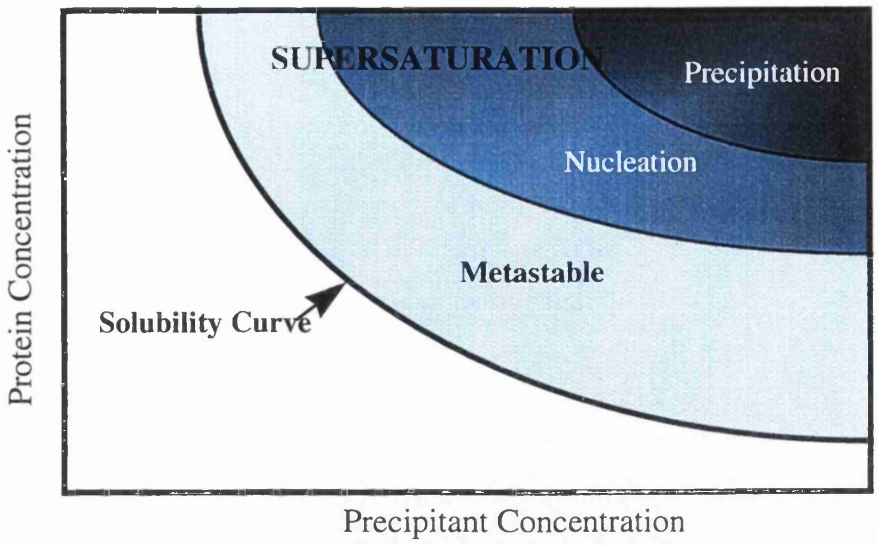


Figure 1: An example of a phase diagram showing different degrees of supersaturation (Ducruix and Geige 1992)

There are three main areas of interest above the solubility curve, the metastable region, the nucleation region and the precipitation region. In the precipitation region, the protein concentration is so high, that it quickly precipitates out of solution as amorphous solid. In the nucleation region the solution will spontaneously form crystals, whereas in the metastable region, crystals will grow, but no nucleation sites will be formed.

When the solution passes the solubility curve, it enters the unstable region of supersaturation. Entering the areas of supersaturation is not enough to form nuclei, the solution must overcome the free energy of germination. Since the area of supersaturation is thermodynamically unstable there will be a resulting energy fluctuation as the solution tries to find a local minima. Sometimes these energy fluctuations are enough to overcome the free energy of germination (Blundell and Johnson 1976). In these cases the protein will undergo a phase transition to a solid forming nuclei. If the energy fluctuations

are not large enough, for example a less supersaturated solution, the solution will not be able surmount this energetic barrier and will only be able to grow on existing crystals. The solubility curve represents the level of saturation, so below this line no crystals will form or grow.

Although this diagram represents the different zones as clear-cut distinct areas, there is quite a bit of overlap. For instance, if nucleation occurs too close to the precipitation region, a shower of microcrystals will result. These borders can also vary with temperature and pH. Looking at the diagram we can see that the ideal situation is to slowly enter the nucleation region, form few nuclei and then move back into the metastable region. Otherwise the protein concentration will be depleted by numerous nucleation sites. If nucleation occurs near the metastable border, the formation of protein aggregates, or nuclei, will deplete the overall protein concentration and drive the solution back into the metastable region to grow a few large crystals. The type of nucleation that can occur is also important to examine. Firstly, primary nucleation occurs when there are no previous crystals in solution. This type of nucleation can happen in two ways: homogeneously when protein molecules aggregate and heterogeneously when foreign objects like fibres, walls, or dust are involved. Secondary nucleation occurs when a nuclei forms onto another previously formed nuclei, usually leading to twinning. As nucleation progresses, it is in a constant state of equilibrium, so as nucleation sites form, some will also dissolve. Aggregates containing lower than a certain number of molecules have a thermodynamically unfavourable free energy and therefore tend to return to solution. Although this critical nucleus may vary it

usually ranges between 10-200 molecules, in general it must be large enough so that the energy of formation from the interactions of involved molecules is negative, and thermodynamically favoured (Blundell and Johnson 1976).

Once nucleation has occurred, the next step is continued growth which proceeds through two stages. First, the protein must undergo a phase transition to come out of solution, and secondly it must correctly orient itself to attach to the crystal. Once a critically sized nucleus has been formed, the molecules build up in parallel planes as the crystal grows. The molecules are thought to build upon a screw dislocation. Consider a nucleation site with a number of molecules in it. Now if a another molecule has sufficient energy to crystallise it will pick the place on the nuclei surface where it will have the most favourable protein-protein contacts. As a result of the higher energy required to start a new layer of molecules, a molecule is unlikely to adsorb to complete layer of molecules. First, two dimensional critical nuclei must form on the crystal surface, leading to surface imperfections. If there is a flaw or a dislocation, then a molecule will be able to establish more favourable contacts within this pocket than if it were to try and attach itself against a complete plane. These defects on the surface lower the energy require for a molecule to affix itself to the crystal (Blundell and Johnson 1976). As new molecules bind to the crystal, they build up spirally around the screw dislocation (Konnert 1996). This type of growth means that there is always an incomplete face to build upon. From this model of growth, it is easy to see how the polycrystalline nature of a crystal arises. As each face is growing, two dimensional nuclei are attaching to the surface, this creates more surface defects

for singular molecules to bind to. This will result in crystal growth in a number of different directions. The resulting crystal lattice will have a number of planes of molecules in it, and not all of these planes will be exactly parallel to one another.

Unfortunately, the final stage of crystallogenesis, the cessation of growth, in crystals is not as well understood as the previous stages. Growth of the crystal will proceed as explained above until a face becomes poisoned or the protein concentration is depleted. It is thought that a face becomes poisoned if there are too many impurities present because they adsorb to the surface. Crystal growth may also terminate if the protein solution falls below the saturation point. Cessation of growth is an important stage because it governs the ultimate size of the crystal (Ducruix and Geige 1992).

CHAPTER 3:

FACTORS OF SOLUBILITY

Having examined the stages of crystallisation, it is also important to look at some of the factors that influence this process. Since proteins are large, complicated, polyvalent molecules (Stryer 1975), one would expect the solutions they are comfortable in might also be quite complicated. The right conditions can make the difference between the formation of amorphous precipitate and crystals. In fact, the complexity of the protein solution provide the subtle differences that induce crystallisation. It is the presence of factors like salts, buffers, cross-linkers, detergents, and organic solvents which influence the protein solubility so that crystals can grow. These factors influence the levels of supersaturation and can help to maintain the appropriate amount of nucleation and growth by stabilising the protein. It is important to remember that although the phase diagram for a protein in solution looks absolute, it is dependant on many factors (McPherson 1982). It is merely a static approximation for the solubility behaviour of protein in solution. Not only do factors in the solution affect the solubility properties, but external factors like temperature and pressure may also play a role. Any factor that influences the solubility of the protein may also affect the crystallisation behaviour of a protein.

As we know, a pre-requisite of crystallisation conditions is to bring a solution to a state of supersaturation. Although there are a number of ways to bring a solution to supersaturation, they all require an understanding of the

solubility behaviour of a protein and the factors that influence it. In general, the solubility of a protein is governed by interactions between the protein and the solvent molecules (Ducruix and Geige 1992). In order for any compound to dissolve in a solution, the strength of the solute-solvent interactions must be greater than or equal to the solute-solute interactions and the solvent-solvent interactions. If the intermolecular forces between the solvent molecules are too strong, the solvent system will not allow the solute molecules to perturb the favourable solvent structure. Similarly, if the solute-solute interactions are strong, then the solute will not want to replace these interactions with solvent interactions and will not dissolve. However, if the solvent-solute forces are similar in magnitude, then the increase in entropy gained by mixing will encourage the solute to dissolve (Holtzclaw 1991). The type of interactions which occur between the protein and solvent can be quite varied: ion-dipole, dipole-dipole, hydrogen bonding, and hydrophobic (Ducruix and Geige 1992). Since most proteins are found in an aqueous environment of the cell, the hydrophobic residues usually pack on the inside of the globular protein and the polar and charged groups are arranged on the surface. As the water molecules surround the protein, they react with this surface. In contrast, membrane proteins are naturally found in the hydrophobic region of the phospholipid membrane. So part of their surface will have the hydrophobic residues exposed to interact with the non-aqueous environment. Despite the origin of the protein, the solubility is a function of the solution and how it interacts with the surface of the protein. Additives to the solutions, such as salts, buffers, detergents, and organic solvents, change the nature of the solution and alter the

solubility. If we have a sound understanding of how these factors influence the solubility, we can use them to manipulate the solution and bring it to a state of supersaturation. For this reason, we must examine factors that will change the character of the solution.

Salt

Owing to the surface charges of proteins, the presence of counter ions in the solution can help to solubilise the protein. As with any charged species, ions are most stable when surrounded by a system of counterions. In a solution of electrolytes, the ions are most stable when surrounded by sphere of counterions. This ionic character perturbs the water structure and changes the solubility of other particles, such as proteins. When an electrolyte is introduced into a solution it dissociates into its ions. How this dissociation affects the solutions is described by a term known as ionic strength. The ionic strength, m, of a solution is defined by the equation:

$$m = \frac{1}{2}\sum cz^2$$

Where c is the concentration and z is the charge or valence of the species. In this way, we can see that since the ionic strength is proportional to the square of the charge a divalent molecule will contribute more to the ionic character of the solution than a monovalent ion. For this reason, we see that many of the common salts used for crystallisation are polyvalent ions, like sulphates or phosphates because they have a dramatic effect on the ionic strength of the solution (Blundell and Johnson 1976).

When considering the effect of electrolytes and protein solubility, there a two major phenomena to examine: salting-in and salting-out. Salting-in describes the fact that most proteins are more soluble in a solution with low levels of electrolytes rather than pure water. Due to the polyvalent nature of proteins, the presence of electrolytes helps to stabilise their solubility by replacing some of the water molecules on the charged surface with counterions. Hence, salting-in is driven by the electrostatic interaction of charged moieties. Salting-out deals with the other extreme of very high ionic strength. When there are many ions in a solution, they are in active competition with the protein and other ions for water molecules. As a result, there are fewer water molecules available to the protein for solvation. Having lost water from the surface, the protein molecules are driven by the hydrophobic effect to aggregate in an attempt to neutralise their surface charges. Unlike salting-in, which is driven by the electrostatic interactions, salting out occurs when the protein minimises the unfavourable interactions on its surface through aggregation. Hence, at high salt (above 0.5M) concentrations the overall solubility of the protein decreases (Ducruix and Geige1992). Although high ionic strength may bring the solution quickly to a state of saturation, it also tends to favour the formation of low solvent containing crystals. Since much of the solvent is taken up with the ions, less solvent makes its way into the crystal lattice and this tends toward greater disorder in the crystal (McPherson 1982). Whether high salt or low salt levels, the presence of electrolytes are able to influence the solubility of the protein by altering the protein-solvent interactions

pH and BUFFERS

The polyvalent nature of proteins often makes their behaviour difficult to predict. Since they are composed of amino acids, they are susceptible to changes in the pH of the solution. Of the twenty amino acids, seven contain ionizable side chains, which depend on the pH of the solution. Each of these side chains has a specific pK_a value. When the pH of the solution is higher than the pK_a, the proton will dissociate from the functional group. For example, the basic amino group of lysine has a pK_a of 10, so the NH_3^+ will not completely dissociate until the solution has a pH higher than ten.

PH of the solution	Ionisation State of Lysine
7	NH_3^+
11	$NH_2 + H^+$

From this example we can see that on either side of the pK_a, the charge of a residue can have different values (Stryer 1975). This means that if a ionisable side chain is on the surface of the protein it can have a variety of charges depending on the proton concentration. This change in net charge can greatly effect the solubility of the protein. Since a protein may contain numerous sites capable of ionisation, it will have a net charge as a unit. The pH at which the overall charge of the protein is zero is known as the isoelectric point, or pI (Stryer 1975). When a protein has a charge of zero, it is least soluble. The proteins can interact with one another, and form a solid without accumulating a net charge as more molecules attach (Blundell and Johnson 1976). This lowers the energy required for aggregation and decreases the solubility.

The pH is not only important for its solubility reasons, but often times a protein will be pH sensitive. It may be more likely to degrade in certain acidic or basic conditions, or it may lose its activity (McPherson 1982). Since the ionisation state of exposed side chains is dependent on the proton concentration, a change in the pH will alter the ionic interactions between protein molecules. New dipole interactions may alter the packing of the molecules and change the resulting morphology and space group of the crystals (Diller 1996). Different unit cells have been produced for only slight pH changes (Northrop 1948). In general, we see that pH alters the solubility of the protein by changing the net charge of the protein. This may effect the activity or even result in a different crystal morphologies (Forsythe 1996). Another problem with buffers is that in order to investigate a whole range of pH, one will have to change buffers. By changing the buffer molecule, one introduces another parameter into the experiment.

Detergents

Although detergents will be discussed in more detail later, they are important tools for altering the solubility of membrane proteins. Unlike soluble proteins, membrane proteins usually have their hydrophobic side chains arranged on the outside and the hydrophilic chains on the inside to minimise energy. Due to the hydrophobic nature of the lipid bilayer, all membrane proteins contain a hydrophobic portion on their exterior surface. Some membrane proteins may have a portion embedded in the membrane and another part protruding into the hydrophilic cell or surrounding aqueous

environment. These large hydrophobic surfaces make the protein sparingly soluble in aqueous solutions. Due to the multiple regions on the surface of the protein, it will not be completely soluble in either a hydrophobic or a hydrophilic environment. This is where detergent molecules fill in the gap of solubility. Detergents are amphiphilic molecules which means they have a portion which is hydrophobic and a portion which is hydrophilic (Ducruix and Geige 1992). They are usually composed of a small polar headgroup and a larger non-polar hydrocarbon tail. Another crucial characteristic of detergents is their ability to form micelles (Lichtenberg 1983). A micelle is a self-assembling circular arrangement that organises all of the hydrophobic tails in the inside and the polar head groups on the outside.

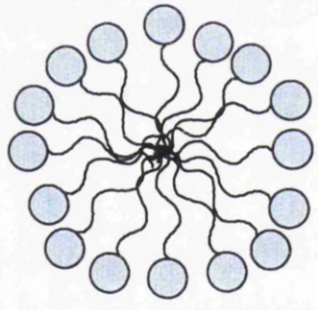


Figure 2. Spherical Micelle

In this way, the membrane protein is solubilised because the detergent molecules associate with the hydrophobic portions of the protein and reduce the solvation energies of these regions. The use of detergent allows one to create a solution that is aqueous in nature to account for the polar region, and simultaneously accommodates the hydrophobic regions with the detergent molecule. In short, the detergent molecule arrange themselves around the hydrophobic regions of a protein, and provide a stable hydrophobic micro-

environment. As the detergents molecules surround the hydrophobic regions, this places their hydrophilic head groups outwards facing the aqueous environment. In this way the solvent is energetically stable, since it is interacting with the polar head group of the detergent molecule while the protein and the non-polar portion of the detergent associate by the hydrophobic effect (Neugebauer 1990).

Organic Solvents

Organic solvents change the solubility in two major ways. Like salt molecules, they compete for the water molecules and secondly they lower the dielectric constant of the solution (McPherson 1982). By competing for the water molecules, the organic solvents leave less water for the solvation of a protein. This competition results in a lower solubility. In order to understand how lowering the dielectric constant affects the solubility, we must briefly examine how pure water solvates macromolecules. The dielectric constant, ε, is a measure of the factor by which a solvent decreases the strength the electrostatic interactions (Ducruix 1982). Due to the polarity and size of water, it is able to extensively interact with the surface of molecules. Since water is competing with solute-solute interactions, its ability to form hydrogen bonds, and weaken the coulombic attraction between protein molecules makes it a good solvent. Let us consider the hydrogen bond that commonly forms between an amide and a carbonyl of a protein:

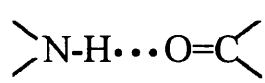


Figure 3: Backbone Hydrogen Bonding

This hydrogen bond stabilises the intermolecular interactions and helps establish tertiary structure. When water is introduced into the system, it diminishes these electrostatic interaction by competing for the hydrogen bonds.

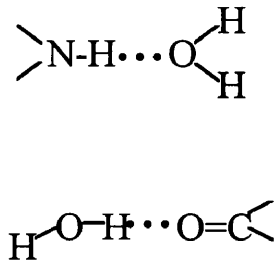


Figure 4: Backbone hydrogen bonding with water molecules

As the water molecules arrange themselves around the charged groups of the protein, these groups are surrounded with a sphere of water molecules. The sphere of water reduces the strength of electrostatic interactions that were available when no water was present. Hence the dielectric constant, ε, reflects the level of depreciation in strength of electrostatic interactions (Stryer 1975). With a dielectric constant of eighty, water reduces the Coulombic attractions of polar molecules by eighty times less than the value of attraction in a vacuum. Water has a very high dielectric constant compared to most other solvents.

Solvent	Dielectric Constant ε
Water	80
Methanol	34
Ethanol	25
Acetone	21

Figure 5: Table of Dielectric Constants (Ducruix and Geige 1992)

If organic solvents are mixed with water the resulting azeotrope will have a dielectric constant lower than water, yet higher than the organic solvent. This results in higher inter-molecular attractions between protein molecules than in pure water. It is easier for a protein to form contacts with other protein molecules because the solvent is now less capable of forming a shell around the charged groups (Blundell and Johnson 1976). With less shielding, the protein molecules are more capable of making stable contacts and hence the solubility is lowered. In general, the dielectric constant reflects the ability of a solvent system to shield the charge species from one another. As the organic solvent molecules perturb the water structure, they lower the solubility of the protein.

There are also a number of drawbacks to using organic solvent. First, organic solvents tend to denature proteins. Solvents like methanol and ethanol interact with the hydrophobic regions of the protein and produce a mild detergent effect. This problem can be avoided by using more gentle organic solvent like MPD or hexanediol (McPherson 1982). If these organic solvents are not effective, then working at lower temperatures can also minimise the denaturing effects (Blundell and Johnson 1976). Another drawback is the volatile nature of many organic solvents. This characteristic becomes particularly troublesome when using vapour diffusion as a means of achieving supersaturation. Experiments like vapour diffusion, depend on the slow equilibration of two liquid solutions, but the addition of volatile organic solvents can speed up the equilibration time. The solution can reach a state of supersaturation too quickly resulting in amorphous precipitate or numerous nucleation sites. In general, organic solvents can be a useful precipitating agent,

but care must be taken to ensure that the protein does not denature or that the solution does not equilibrate too quickly.

Temperature

Although crystallisations have been reported from a range of 0-40 °C, most crystallisations are set up at 4 °C and 25 °C, corresponding to cold room and room temperatures. Despite the temperature chosen, it is important to keep the solutions at a stable temperature. Slight changes in temperature will alter the solubility and may effect the crystallisation behaviour. If the solubility of a protein is well documented, one may even use this change in solubility to induce crystallisation (Blundell and Johnson 1976). Like most of these factors, temperature affects the solubility by changing both the solvent and the solute interactions. The temperature has an inverse relationship on the dielectric constant, that is, as the temperature increases the dielectric constant goes down (Blundell and Johnson 1976). As the temperature increases, the solvent molecules will undergo more Brownian motion. This leads to an increase in the disorder of the system. The increase in free energy of the system, means the solvent molecules are less able to maintain the solvent shell around polar entities. Hence the dielectric constant of the solutions will go down as the temperature increases. The increase in free energy of the solution will also effect the solubility of the protein molecules. The disorder of the solvent molecules makes it easier for the protein to be solvated, making it is easier for the protein molecules to break into the solvent structure and dissolve.

The affect of temperature can also be influenced by other factors, like salt. At low salt concentrations, the solubility effects of both temperature and pH are both amplified. In fact using low ionic strength conditions has recently been used to investigate the effect of pH and temperature for crystallisation (Harris 1995). Whereas at high salt concentrations, most proteins are more soluble at lower temperatures (Blundell and Johnson 1976). In this way we can see the influence of temperature may depend on other properties of the solution, such as ion concentration. Changes in temperature may also change the morphology of the crystals. For instance, lysozyme grows as tetragonal crystals at low temperature, while above 25°C orthogonal crystals are favoured (Jolles 1972). This happens because the change in temperature shifts the place on the phase diagram at which nucleation and growth occur. Although temperature effects the solubility and habits of crystallisation, one must be careful that the temperature does not cause the protein to denature. At high temperature, the intramolecular bonds that maintain the activity and tertiary structure of the protein may break down resulting in denatured protein (Stryer 1975).

CHAPTER 4.

HETEROGENEITY

Having examined some of the factors that influence the solubility of a protein, we can now address another issue which permeates all phases of the crystallisation process, homogeneity. Homogeneity of the solution is important because it promotes homogeneity and order in the crystals. Statistically speaking, one population of molecules in solution is more likely to produce a homogenous crystal. When the crystal is forming and molecules are coming out of solution, the presence of two or more populations increases the likelihood of non-homogeneous crystal formation. This heterogeneity can lead to higher disorder in the crystal and thereby decrease the overall quality of the crystal (Thomas 1996).

Non-homogeneity in the solution causes a number of problems by interfering with the normal nucleation process. By providing nucleation sites, the presence of other molecules lowers the energy barrier for nucleation (Ducruix and Geige 1992). This is known as heterogeneous nucleation because different molecules serve as the nucleation site to which protein molecules can add (McPherson 1982). This nucleation will influence the solubility of the protein because it may cause premature and numerous crystal growth. Unlike a homogenous system, the solution will not have to enter the nucleation region of supersaturation to generate nucleation sites. This will also disrupt the kinetics of the process of crystal growth. The solution does not have to equilibrate to a point of nucleation, form nuclei and then pass back into the

metastable region to grow. Instead, these heterogeneities in solution provide nucleation site. So when the solution makes what would have been its first pass through the metastable region, crystals will grow. Changing the kinetics of the equilibration may encourage disorder in the crystal, because the molecules may not have enough time to orient themselves in the correct position in the lattice. These small defects will compound one another and cause irregularity.

Heterogeneity in the solution can also interfere during other stages of crystal growth. As the foreign species work their way into a previously homogenous crystal they serve as secondary nucleation sites. Secondary nucleation often leads to growth of another crystal lattice in an opposing direction and eventually to a twinned crystal. The final complication of heterogeneity in a solution for crystallisation is that the presence of impurities can poison a face of a crystal and terminate further growth (Ducruix and Geige 1992). From these examples of how heterogeneity can effect crystal growth during all stages of crystallogenesis, let us now examine some common sources of heterogeneity.

Heterogeneity in a solution can arise from any number of places. It can be the presence of dust or particulate matter, degraded protein, or even an additive such as detergent or glycerol. Foreign matter like dust and fibres can usually be minimised by centrifuging the sample (Ducruix and Geige 1992). Then the top of the sample is removed and used for crystallisation trials while most of the dust and particulate matter remains in the bottom of the original sample vial. Foreign sources, like dust, are more easily minimised than internal

sources. When considering homogeneity of a solution, we want all the molecules to be as identical as possible to ensure regularity in the crystal. This includes aspects of the molecules like charge and covalent modifications (McPherson 1982). For this reason, it is important to avoid pH or salt conditions where the charge of a protein is oscillating between charge states. By far, the most common source of heterogeneity in a solution is the degradation of the protein itself. These degradation products are difficult to identify and eliminate. Denatured protein has adverse effects because it is less stable the native protein, so it is more inclined to precipitate out and provide unwelcome nucleation sites. The use of harsh organic solvents, high temperatures, and lyophilization can generate denaturation products and should be avoided (Blundell and Johnson 1976) The level of denaturation can be monitored by a number of methods. Denatured protein may lose activity due to a conformational change. Employing spectroscopic techniques, like circular dichroism which monitor the protein conformation, may help to detect the level of denaturation (McPherson 1982).

One source of heterogeneity is proteolytic cleavage. If there are proteases present during the purification process, they might have begun to degrade some of the protein molecules. This occurs in two major ways, they can cleave amino acid residues from the carboxy or amino termini or they chop the protein at a specific sequence. In some cases the protein may still remain associated, but in other cases it may result in a solution composed of various protein fragments. Proteolytic cleavage can be controlled by different approaches. One can introduce protease inhibitors to the system to minimise

the amount of degradation (McPherson 1982). If this approach is taken, one must remember that these enzymes and inhibitors will introduce another parameter to worry about during crystallisation. Alternatively, one can use proteolytic cleavage to one's advantage, by using controllable proteases to produce uniform fragments of known size (Steitz 1981).

Another source of heterogeneity in the solution might be the nature of the protein itself. Slight physical differences in a protein population can manifest themselves in a number of ways. They can originate from the expression system or from post-translational modifications, such as glycosylation. For example, the coding for certain proteins may contain point mutations leading to a sequence alterations or even modifications of certain amino acids. The cell line *Bacillus brevis*, is used to manufacture the polypeptide gramicidin (Hotchkiss and Dubos 1940). Despite the same genetic sequences the system produces three different isoforms varying between tryptophan, phenylalanine and tyrosine at the eleventh residue (Stankovic 1990). In this case, it is best to isolate one of the most plentiful isoforms and use that population. Similarly, glycoproteins often have a great deal of variation in the size of their carbohydrate. So in order to produce a homogeneous solution, the carbohydrate is often removed. Yet another problem that may result from the expression system is the covalent modification of certain residues. Methylation, amidation, phosphorylation, and hydroxylation may only be slight changes, but the introduction of a large charged group, like a phosphate will influence the charge state of the protein surface (McPherson 1982).

Another major problem of heterogeneity is the problem of aggregation states. Depending on the solubility of the protein, it may be more stable when aggregated as a dimer, trimer or *n*-mer. This would be reasonable if the whole system was in a dimer state resulting in homogenous population, but if the solution has multiple aggregation states it becomes difficult. To resolve this complication, one should reduce the whole system to a minimum state of aggregation. Most commonly this can be achieved through manipulating the pH and the electrolytes. Using these parameters, one can alter the charges on the ionizable amino acids. These modifications of the protein surface may adjust the strength of the protein-protein interactions which hold the aggregates together. The influence of divalent cations has been shown to influence the aggregation state. It is thought that ions like Ca^{2+}, Cd^{2+}, or Zn^{2+} are able to bridge negative groups on the same molecule and in a sense, prevent this site from being used to link to another protein molecule (McPherson 1982). Detergents can also be used if the attraction of molecules in mainly motivated by hydrophobic interactions. The aggregation state of a protein in solution can be examined through particle sizing techniques like dynamic light scattering (Malkin 1994) or ultra centrifugation (Ralston 1993). In fact the use of dynamic light scattering has gained increasing popularity as a pre-crystallisation screening technique due to its ability to use a small, dilute and recoverable sample to monitor the aggregation state of the protein (George 1994). If the solution gives a maximised reading, which reflects a single sized population, it is more likely to crystallise (Veesler 1994). Overall, it is clear that a homogenous solution is an important consideration when trying to

produce well-ordered, diffraction quality crystal. Although homogeneity is not an absolute requirement, in most cases, it can increase the statistical chances of producing diffraction quality crystals. Whether the heterogeneity results from denaturation, degradation, foreign matter, aggregation or physical differences in the protein molecules, it should be minimised to assist the crystal formation.

DYNAMIC LIGHT SCATTERING

An excellent measure of the heterogeneity of a given solution can be obtained from dynamic light scattering. In recent years, the polydispersity of a sample has been linked to the quality of crystals grown for x-ray structural analysis (Veesler 1994). Dynamic light scattering employs the idea that when monochromatic light passes through a solution, the light is scattered in all directions by the molecules within the solution. By measuring the amount of scattered light, we can determine information such as molecular weight, polydispersion, hydrodynamic radius and estimated shape.

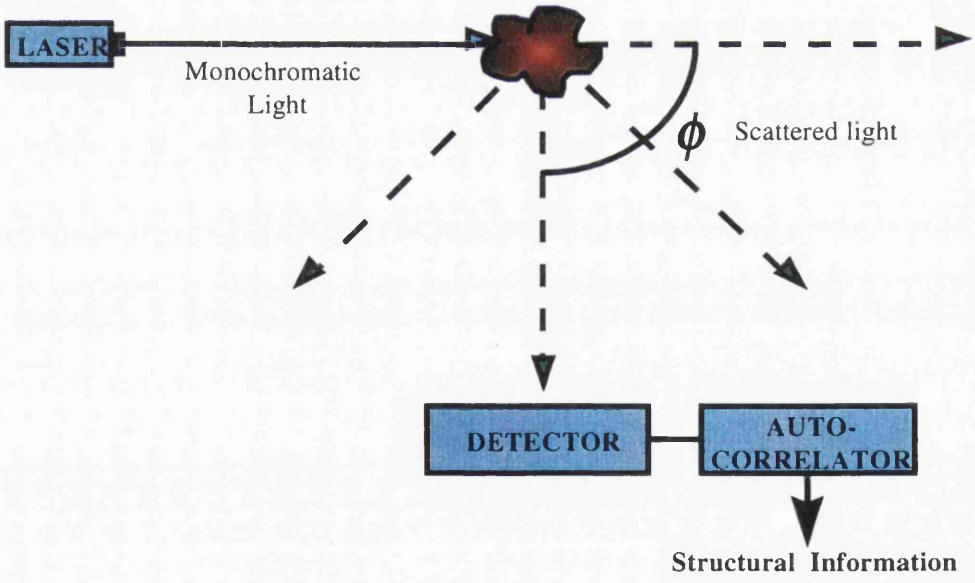


Figure 6: Schematic representation of a light scattering instrument. The monochromatic light is scattered by a molecule in solution and collected by the detector at the scattering angle ϕ. The photons are recorded, transferred into electronic impulses, and then statistically processed by the autocorrelator to yield structural information.

There are two major ways of looking at what happens when a solution is illuminated by monochromatic light, one from the view of the molecules in solution and the other from the view of the photons in the light. In general, when a molecule is in a field of electromagnetic radiation, a molecule will either absorb or lose energy. How the molecule reacts in such a field can provide useful information. For example, in dynamic light scattering when light strikes a solution, it induces an oscillating polarisation of the electrons of the molecules. The oscillation of this induced dipole creates another electric field, known as scattered light, which may be out of phase or in a different direction to that of the original light source. If size of the molecule illuminated is less than five percent of the wavelength of original light, then the intensity of the radiated light for a single molecule can be predicted by the Raleigh scattering relation (Berne 1976):

$$I_{s,o} = \frac{4\pi^2 M^2 \sin^2\phi \, (dn/dc)^2 I_o}{N_A^2 \, \lambda^2 \, r^2}$$

Where M is molecular mass in Daltons, ϕ is the scattering angle measured between incident beam and the scattered light, dn/dc is the dependence on solute concentration, I_o is the intensity of the original beam, N_A is Avagadro's number, λ is the wavelength of the original beam, and r is the distance from the molecule to the detector.

From the other perspective, as the photon passes through the solution, it will gain or transfer energy to the translational, rotational, vibrational, electronic degrees of freedom of the molecule. However under the Raleigh

scattering theory, our considerations are limited to light scattered from translational and rotational degrees of freedom (Berne 1976). This change in the photon's energy can cause the frequency of scattered light to be different than the original beam. The frequency shift, intensity and angular distribution depend on factors like size, geometry, and interactions of the molecules. For dilute solutions of particles such as proteins, the amount of scattered photons is actually very small, of the order of thousands of photons. In fact, the low level of scattered light was the limiting factor in light scattering technology for a number of years. Previous to 1964, the monochromators available were unable to resolve the small frequency shifts of the scattered light. However the use of lasers by Pecora provided an intense source of monochromatic light which eases the detection of scattered light and creates resolvable frequency distribution (Pecora 1964).

Having overcome the problem of detecting the weak signal, a further complication arises from the nature of the sample solutions. First, the solution is not made up of a single scattering molecule, but a great number of them. Secondly, all of these particles are in a constant state of fluid dynamic movement, leading to variation in the amount of light scattered. This means that the detected signal is actually a compilation of all scattered light from a number of molecules. For a solution with many particles in it, the intensity of scattered light will vary due to the Brownian motion of the solution. As the particles diffuse around the solution, the intensity of the scattered light will vary with the distance from the detector. This translation of the molecules,

leads to fluctuations in the recorded scattered light intensity. As a result of this Brownian motion, each molecule's contributions to the total intensity will vary depending on its phase. Hence the resulting signal will reflect this summation, causing constructive and destructive interference within the signal over time. The resulting time dependant shifts in frequency yield information about the dynamics of the molecule (Parker 1997). The detector, usually a photo-diode, reads the time dependant fluctuations in intensity, centred around a time-averaged intensity. So if we describe the instantaneous intensity of scattered light as

$$I_s = I_{s,o}\left(N + 2\sum_{j>i=1}^{N} \cos\left(\delta_i - \delta_j\right)\right)$$

Where $I_{s,o}$ is the fundamental scattering intensity for a single molecule, N is the number of scattering molecules, and the δ terms represent the phases from the ith and jth particle. This means there will be a time averaged scattering value described by the first term and the second term accounts for the short term fluctuation. Since all the waves of scattered light are detected as one signal, they act statistically the same as one molecule that scatters the total intensity of light and diffuses over time. As this imaginary singular scatterer diffuses about the solution, there will be slight variation in the intensity of scattered light as it varies its distance from the detector. This accounts for the decay in the signal about the average intensity. It is from this short term

fluctuation that we can determine the value of the hydrodynamic radius, using the statistical tool known as autocorrelation.

Autocorrelation was originally developed as a time dependant statistical analysis to help resolve signal from noise. In order to get a time dependant function, the photodiode takes a sampling at a specific interval of time small enough to ensure that the slight fluctuations in average intensity of scattered light are detected. This value is known as τ, or the decay time. Using the autocorrelation function to interpret the time dependant signal, we can determine the value of the decay time, τ. The mathematics involved in solving the autocorrelation function are quite complicated, but the basic formula is (Parker 1997):

$$\lim_{T\to\infty} \; 1/T \int_{\tau}^{T} I(t)\, I(t+\tau)\, dt,$$

Where T is the total time sampled and I(t) is the intensity at a given time. Once this equation is solved, we can use the decay time to determine D_T, the Diffusion coefficient and then use the Stokes Einstein equation:

$$R_H = \frac{k_b T}{6\pi \eta D_T}$$

Where R_H is the hydrodynamic radius, k_b is Boltzman's constant, T is the temperature in degrees Kelvin and η is the solvent viscosity. The average hydrodynamic radius provides information about the size and shape of the molecule. So overall, the monochromatic light is scattered by the particles in

solution, producing a signal of time dependant fluctuations due the different positions of each molecule and their Brownian motion. Using the autocorrelation function, one can obtain the decay time of the signal and use this value to determine the diffusion coefficient, hydrodynamic radius and polydispersity (Berne 1976).

The polydispersity measurement is a statistical measurement that reflects the standard deviation of the spread of particle size about an average radius. With the polydispersity value, one can use dynamic light scattering to monitor the state of aggregation of the protein in a given set of conditions. In an ideal protein solution for crystallisation, the solution will slowly be brought to a state of supersaturation, then a few nucleation site will form resulting in a few large crystals. In fact, this is rarely the case. Often the protein forms aggregates, or random formations of proteins. These aggregates are classed into two groups, craggs and praggs. Craggs are aggregates which lead to crystals, whereas praggs are aggregates which lead to precipitation (George 1994) Although light scattering cannot differentiate between the two, it can identify the presence of aggregates quite clearly. As there are more aggregates in solution, there will be a larger Gaussian distribution around the average hydrodynamic radius, this will lead to an increase in the polydispersity. By analysing the distribution of hydrodynamic radius it is also possible to determine whether the aggregates are dimers, trimers and so on. Because the state of protein aggregation can greatly influence the crystallisation of a protein, dynamic light scattering is becoming a powerful pre-crystallisation

technique. According to George, the pre-crystallisation methods should fit the following criterion (George 1994). First the technique should produce a reproducible discriminating result, that defines one range that yields crystals and another range that will not. Next, the technique should require a small amount of protein, in any solvent, and be non-destructive. Finally the technique should be simple enough to be performed routinely in the laboratory. Dynamic light scattering fits this criteria very well. The monodispersity of the sample is indicative of crytallisibility under a given set of conditions. Each run only takes about 300 μl usually ranging between 1-10mg/ml depending on the molecular weight of the protein, which can also be recollected at the end of the sample. And the technique is simple enough to be set up in any laboratory. Hence, dynamic light scattering is a powerful pre-crystallisation technique. Through the polydispersion value one can investigate the aggregation state of the protein in a given set of conditions to determine whether setting up crystallisation trials will be worthwhile, thus saving time, effort and valuable protein (Malkin 1994).

CHAPTER 6

SEEDING

In recent years, seeding has become an important technique during the process of protein crystallisation. It has been shown to be an efficient, reproducible technique for enlarging small crystals to large diffraction quality crystals (Scheffzek 1994). Seeding has been successful in a number of proteins (Spinelli 1996) and complexes (Stura 1994) as well as been incorporated into systems of pre-crystallisation screens (Stura *Reverse* 1994). In addition, seeding can also be used to distinguish between amorphous precipitate or microcrystals (Ducruix and Geige 1992). In short, seeding techniques are quickly becoming an integral tool in protein crystallography to overcome some of the obstacles of the crystallisation process, and deserve a more detailed look.

Considering all the complications that may result from the affiliation of nucleation and continued growth, it is easy to see why a technique that uncouples these two stages would be desirable. There are many difficulties that originate during the nucleation stage. Partially ordered nuclei may lead to badly ordered crystals. Too many nucleation sites may produce microcrystals or encourage twinning, while too high a level of supersaturation will cause rapid growth and result in disordered crystals. Many such problems can be minimised or avoided through the separation of nucleation from continued growth. Since we have previously examined the procedure of crystal growth, we should understand its dependence on the solubility behaviour of the protein and its significance throughout crystal growth. Usually the protein solution

must be brought to a level of supersaturation that will allow for spontaneous nucleation and then proceed to grow at the lower, metastable level of supersaturation. As one can imagine, finding the perfect conditions that foster this situation can be very difficult. The use of seeds in crystallisation provides pre-formed nuclei that serve as a template for crystal growth, and thereby avoids the nucleation region and the pitfalls associated with it. In a system that often has many different parameters, anything that can reduce the number of conditions to be manipulated is well appreciated. In this way, seeding uncouples the process of nucleation from crystal growth, allowing us to focus on the adjustment of conditions to optimise growth.

Seeding has become an important technique in crystallisation because it provides a method for the production of large reproducible crystals. The introduction of seeds into a solution provides well-ordered nuclei on which protein molecule can build upon and form large crystals. There are two major techniques of seeding - macroseeding and microseeding. Macroseeding introduces large crystals into a system, whereas microseeding only uses very small seeds. Whichever method is chosen, the stages of the process are still the same. Seeding techniques can be broken down into three major areas: pre-seeding, analytical seeding and refinement of growth conditions (Ducruix and Geige 1992).

Pre-seeding includes all the conditions that influence the conditions of the solution prior to the introduction of seeds. Ideally, the solution only needs to be brought to a metastable region of supersaturation, and then will spontaneously grow with the introduction of seed crystals. Since the

conditions to bring the solution to a metastable region will be different from those which spontaneously grow crystals, one will need to investigate which level of supersaturation responds best to seeding. This is usually found through an analytical method like streak-seeding. For streak-seeding, a probe, usually an animal whisker, is dipped in a seed-containing solution and then streaked through the pre-equilibrated well to determine the appropriate level of saturation. Animal whiskers are the best probes because they consistently deliver seed to 6-12 drops before needing to be re-dipped in seed solution (Ducruix and Geige 1992). The probe actually absorbs some of the seeds from a seed solution or from rubbing a crystal face and then deposits some of the seeds in the progressive solution. Due to the nature of the whisker, only a few seeds are transferred on each dip, keeping the number of nuclei to a minimum. When a probe becomes too old, it deposits all its seeds in the first couple of drops and then none after that. This can usually be seen from the resulting growth in seeded wells. The level of supersaturation that responds best may vary with factors including, protein concentration precipitant concentration as well as level of equilibration. One can test which stage of equilibration is the most beneficial to growth by introducing seeds at different times during the equilibration of the wells. Certain times will respond better, and they should be used for further experiments.

During the process of analytical seeding, one investigates the levels of supersaturation that provide the optimal conditions for further growth. Usually, a range of conditions are set up at varying protein and precipitant concentrations to screen for the best combination. The probe is passed,

perpendicular to the surface of the equilibrated well, once. Depending on the results of this type of experiment, one can determine the best conditions for seeding. For example if no crystals form, then the solution was not saturated and the seed crystals probably dissolved because the precipitant level was not concentrated enough. If the concentration of precipitant was too high then crystals will initially form along the line of streaking and then spontaneous nucleation will occur away from the line. The optimal level of precipitant concentration will produce crystals only along the line.

Having found the approximate level of supersaturation through streak-seeding, these conditions can further be refined and adapted to the production of diffraction quality crystals. According to Stura (1994), you want to increase the drop size while decreasing the precipitant/protein ratio and precipitant concentration of the reservoir. These changes should help to slow down the equilibration process. Once these optimal levels have been found, you can proceed with either method of production seeding, micro seeding and macroseeding.

In microseeding, a stock solution of microseeds is prepared by crushing up crystals and then diluting them a number of times. Each of these solutions is tested by streak seeding to see which one delivers the optimum amount of crystals. Unlike streak-seeding for the purpose of finding optimal supersaturation levels, for crystal growth you do not want a complete line of crystals but only a few seeds. This favours the growth of larger crystals. In general the procedure is as follows. Once the crystals have been crushed they are usually diluted between 10^3 and 10^7 times (Ducruix and Geige 1992). To

find the minimum level of precipitant, some drops are streaked at the level found prior, then the reservoir solution is diluted to differing levels. This change will cause some of the streaks to dissolve, and the streaks that remain will indicate the minimum level of precipitant necessary to maintain the crystals. Then precipitant is added to the seed solution to make it up to this minimum concentration of precipitant. Now the seeded solution can be used for further seeding. Although the technique is the same as for analytical seeding , there a few points that should be addressed. First, the same whisker should be used for consistency. The size of the whisker may also effect the size of the crystal seeds delivered. Larger whiskers have bigger pores so can carry larger seeds. Since the probe will carry whiskers after each streaking, it does not need to be re-dipped after each streak. Further dilution can be achieved by dipping the probe into the reservoir prior to streaking the drop. To reduce evaporation, air exposure for both the probe and the drop must be kept to a minimum. Most seeding results develop much quicker than normal vapour diffusion experiments, only a few days to a week in most cases (Stura *Reverse* 1994).

In macroseeding, as the name implies, one prepares a pre-equilibrated solution and then places a single well-formed crystal into the solution. Since the surface of the crystals often contains defects or impurities that stopped its growth in the first place, the crystals should be rinsed in a slightly dissolving solution prior to their introduction to the seeded solution. Washing away the surfaces will help to provide new places for protein to attach to and encourage crystal growth. Assuming the crystal has maintained its integrity, it can be

moved to stabilising solutions of the original mother liquor solution. This will remove the original mother liquor and help reduces the chance of smaller seed being transfer. It will also minimise the amount of transferred protein from the surrounding mother liquor. The addition of protein in soluble form may disturb the equilibrium of the seeded drop and change the seeding behaviour. After about four washes, the crystal can be transferred to the drop to be seeded and hopefully continue to grow (Ducruix and Geige 1992). This procedure can be done a number of times until the crystal grows to a reasonable size (Sivaraman 1996).

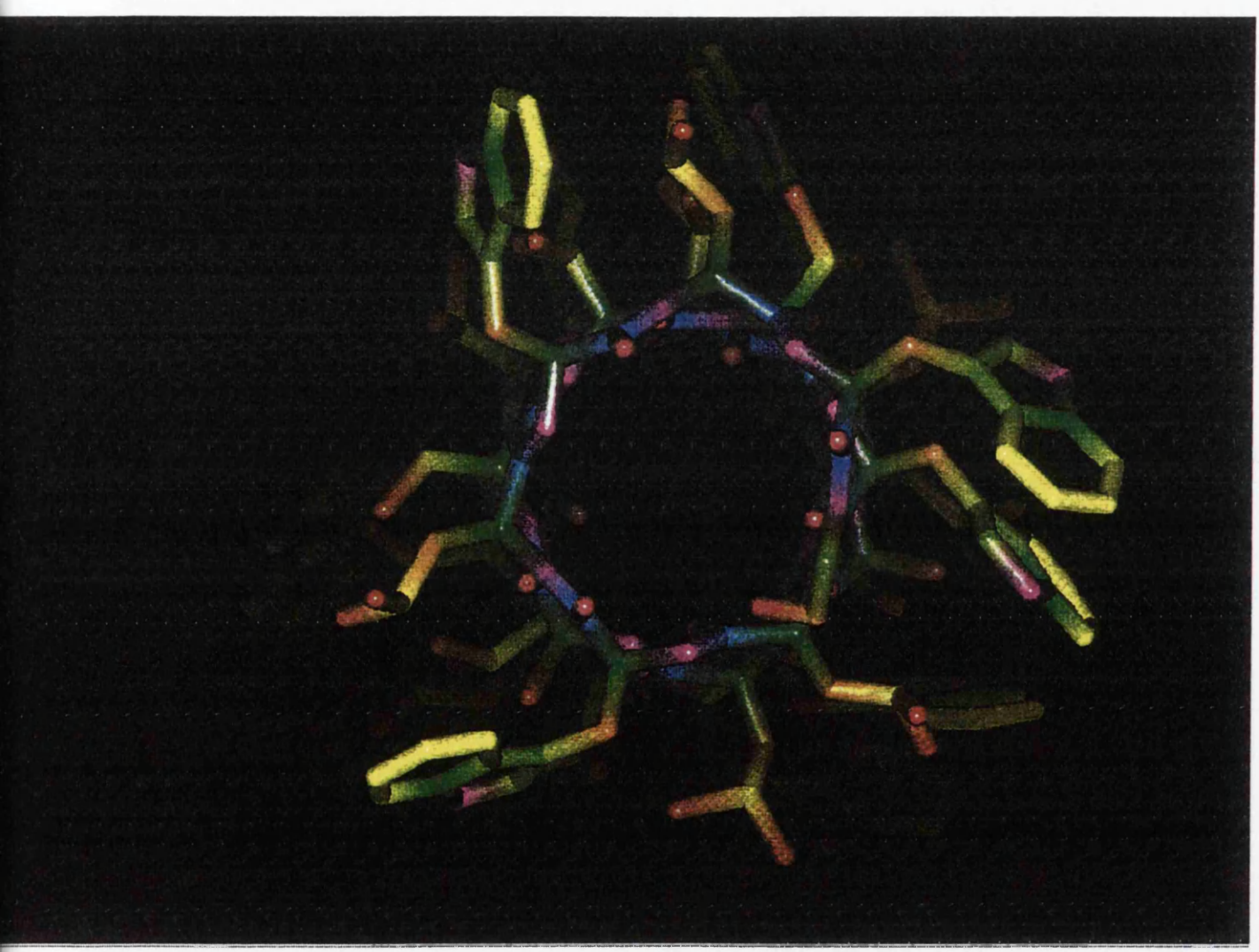


Figure 11. View down a gramicidin helix monomer. Note the central 4 Angstrom hydrophilic pore and the hydrophobic exterior made up of bulky side chains. When in the membrane, these groups extend into the hydrophobic region (Image site).

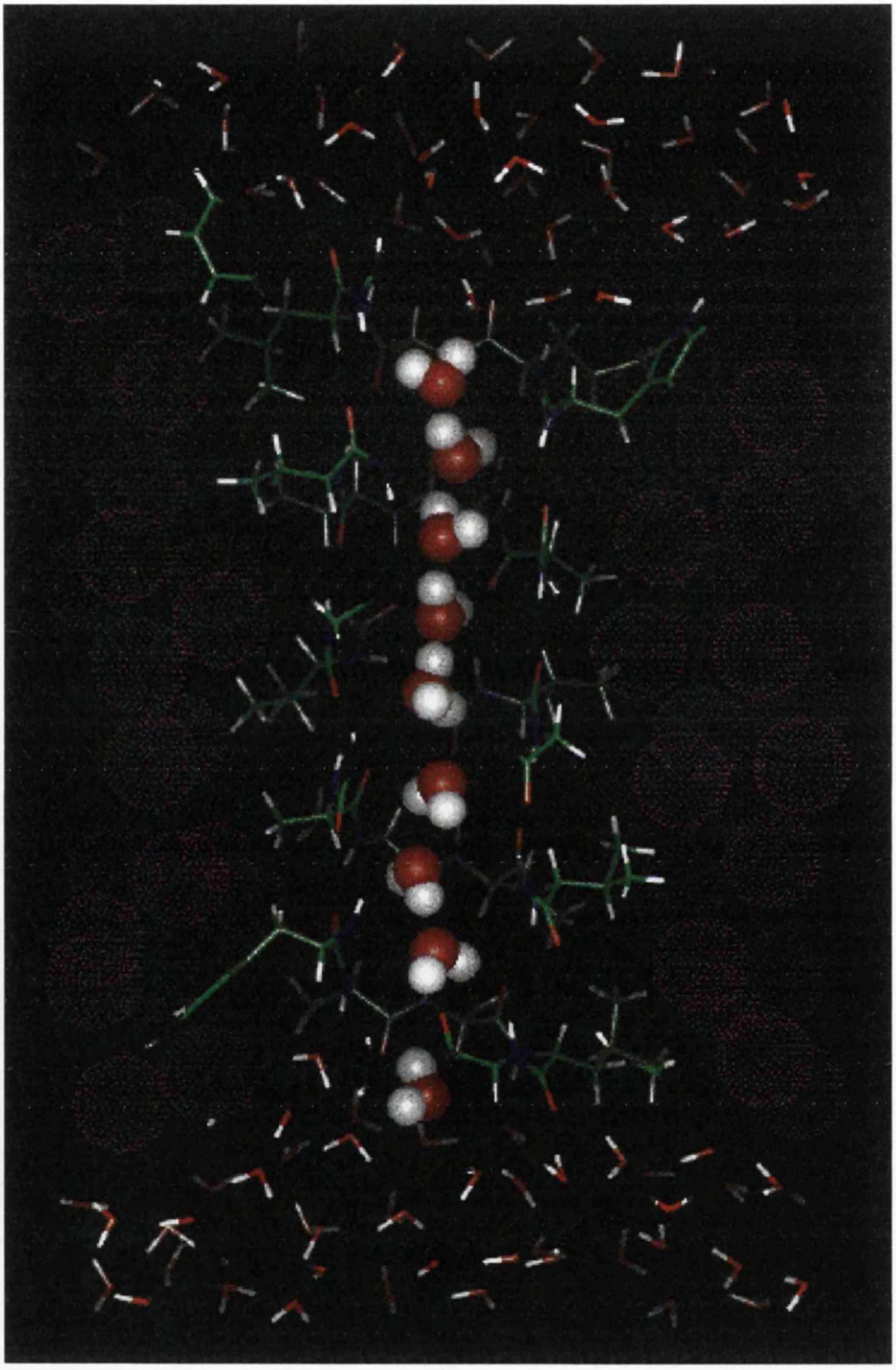


Figure 13. View along two associated gramicidin monomers (green). Also note the water molecules in red and white. The purple represents a hydrophobic surface. The dimer is approximately 25 Angstroms in length (Dimer site).

Figure 19. Example of a streak-seeded well. Microcrystals contain gramicidin and LDAO detergent.

Figure 21. Shovel-shaped crystals of gramicidin A and LDAO detergent in methanol and water

PART II:

GRAMICIDIN A

CHAPTER 7.

INTRODUCTION

This study will focus on the crystallisation of gramicidin A, a peptide antibiotic which forms a membrane spanning channel. Gramicidin A is the best characterised of all the ion channels. Although, the channel has been well studied for its conductance, covalent modifications, and other characteristics, its structure solution via crystallography has remained somewhat elusive. Like most membrane proteins, gramicidin is not easily crystallised. Therefore, researchers have used other methods, such as modelling (Urry 1971), mutagenesis (Barrett 1986) and CD (Massotti 1980) to propose a structure. Despite the large amount of information available on gramicidin, no crystal structure exists that reflects the structure of the conducting dimer in its natural lipid environment. A crystal structure of gramicidin A in its head to head channel form would be extremely valuable since it would clarify previous models. This leads us to the very important question of why gramicidin has proved so difficult to crystallise. Since gramicidin is a membrane protein, let me first review the importance and difficulties of working with membrane proteins, then discuss the detergents role as a additive to produce membrane protein crystals, and finally discuss how this pertains to the case study of gramicidin A

CHAPTER 8

MEMBRANE PROTEINS

All life takes place on either side of a membrane. A cell is dependent on its membrane's ability to regulate gradients of salts, nutrients, and substrates. This membrane is composed of a lipid bilayer impermeable to most particles and ions. In order for a cell to obtain the necessary materials for life, it has a complex system of proteins that move ions and substrates across the membrane. These enable the cell to maintain the electrochemical gradient necessary for proper function.

An example of the importance of the electrochemical gradient is the way a nerve cell propagates a signal. The impulse which travels down the nerve fibre is a wave of ionic fluxes. Each nerve cell has a resting potential that is determined by the distribution of ions across its surface. Through the membrane-bound Na-K pump, the cell maintains the appropriate concentration of K^+ and Na^+ levels within the cell. The membrane has a resting potential of about -60mV, very close to the K^+ equilibrium potential of -75mV. This results in the membrane being more permeable to K^+ ions at the resting potential. Upon depolarisation, the action potential is mediated by gated ion channels. When the axon depolarises, this triggers the opening of sodium channels in the membrane. This allows Na^+ ions to flow into the interior of the cell, and signals the opening of even more sodium channels. Because of the high concentration of Na^+ ions outside the cell, the flow of Na^+ ions is thermodynamically favoured until the membrane potential reaches +30mV, the Na^+ equilibrium potential. At this point, the sodium-pumps

close and the Na-K pump begins to pump the Na^+ back out to regenerate the -60mV resting potential. Hence, channels and membrane spanning complexes play a crucial role in processes such as signal propagation through the manipulation of the electrochemical gradient across a cell membrane (Stryer 1975).

In fact, their role is so crucial to life function that aberrant channel function has been implicated in a number of very severe pathophysiological, and often lethal disorders in a variety of organs. For instance, Cystic fibrosis, the most common fatal genetic disease in the United States, has been linked to defective channel transport of sodium and chloride ions in epithelial cells. These epithelial cells are responsible for lining the outer surfaces of organs like the lungs and pancreas. Abnormal Cystic fibrosis cells produce a thick mucus that clogs up the organs, resulting in lung infections and reduces enzyme flow from the pancreas. Because the enzymes cannot get to the stomach, patients cannot absorb all the nutrients they consume and suffer from malnutrition. This symptom, coupled with the high risk of lung infection leads to an average life span of only thirty years (Cystic Fibrosis Foundation 1997). Even a simple point mutations of ion channels can lead to heritable neuromuscular disorders such as familial periodic paralysis and myotonia (Cannon 1996). Abnormalities in the electrical excitability of the muscle fibre lead to sustained muscle contractions. These alterations in excitability arise from mutations in voltage-gated ion channels which control chloride, sodium, and calcium transport (Hayward 1997). In addition, defective sodium channels have been found to contribute to the demyelination states of multiple schlerosis patients (Dugandzija-Novakovic 1995). Finally, the neurotoxic

effect of Alzheimer's disease may be related to the channel forming capabilities of the beta amyloid protein (Arispe 1993). These cases show that despite the type of tissues, when a cell loses its ability to regulate the delicate electrochemical gradient, the effects are very severe.

From these numerous examples, it is clear that dysfunctional or inactive channels can have severely debilitating effects on cell function. When a cell can not maintain and respond to changes in the electrochemical gradient, it is unable to function and communicate normally. Ion channels, such as gramicidin, belong to a class of compounds which transport and redistribute charges ions across the membrane. Ion channels are a subclass of compounds known as transport antibiotics. Transport antibiotics are molecules that allow charged particles to cross the membrane and they are generally classified by the mechanism by which they move the ion. First, carrier molecules engulf the ion, pass through the hydrophobic region of the membrane and then release the ion on the other side (Stryer 1975). In general, carriers, often a pump system, require some form of energy to drive the system because they usually work against the electrochemical gradient. On the other hand, ion channels span the membrane and produce a hydrophilic channel through which the ion can traverse the membrane. Channels usually work in favour of the gradient and therefore require no additional energy. In short, both types of transport molecules make the membrane permeable to charged particles, although through different means.

Transport antibiotics also fall under the larger class of membrane proteins. Although membrane proteins make up more than half of all proteins, this

percentage is not reflected in the number of crystal structures (Michel 1991). In fact, most of the protein structures published describe soluble proteins. This discrepancy is a direct result of the difficulties of crystallising membrane proteins. Since they are associated with the membrane they each have unique solubility characteristics, making them more difficult to crystallise.

CHAPTER 9:

DETERGENTS

The unique solubility characteristics of membrane proteins has led to the breakthrough use of detergents during the crystallisation process. Since membrane proteins are usually partially or completely imbedded within the cell membrane, certain portions of their surfaces are hydrophobic while other exposed portions are hydrophilic. During crystallisation, detergent molecules interact with the hydrophobic surfaces of proteins to help prevent aggregation. Although this role of detergents in crystallisations may seem fairly straight forward, finding the right conditions often proves more difficult. In order to maximise the effectiveness of detergents, it is necessary to have a good understanding of these molecules and how they behave in solution.

Detergents are a subclass of molecules called amphiphiles. The amphiphilic characteristic of detergents makes them particularly useful when working with membrane proteins, as they are able to mimic the hydrophobic and hydrophilic environment that a protein would usually experience in the phospholipid cell membrane. Amphiphilic molecules contain both a hydrophobic and hydrophilic region within the same molecule. The hydrophobic end of the molecule is usually composed of an aliphatic chain under twelve carbons (Ducruix and Geige 1992), while the hydrophilic portion can be a number of functional groups ranging from charged anionic, cationic, or zwitterionic to uncharged polar groups such as polyoxyethylene. This allows for a huge range of combinations of head groups

and tails and results in a varied population of amphiphiles. There are two major classes of amphiphiles, insoluble and soluble. Insoluble amphiphiles, such as phospholipids and tri-acyl glycerols form stable monolayers or lamellar liquid crystals in aqueous solution. On the other hand, detergents form unstable monolayers and are able to form micelles.

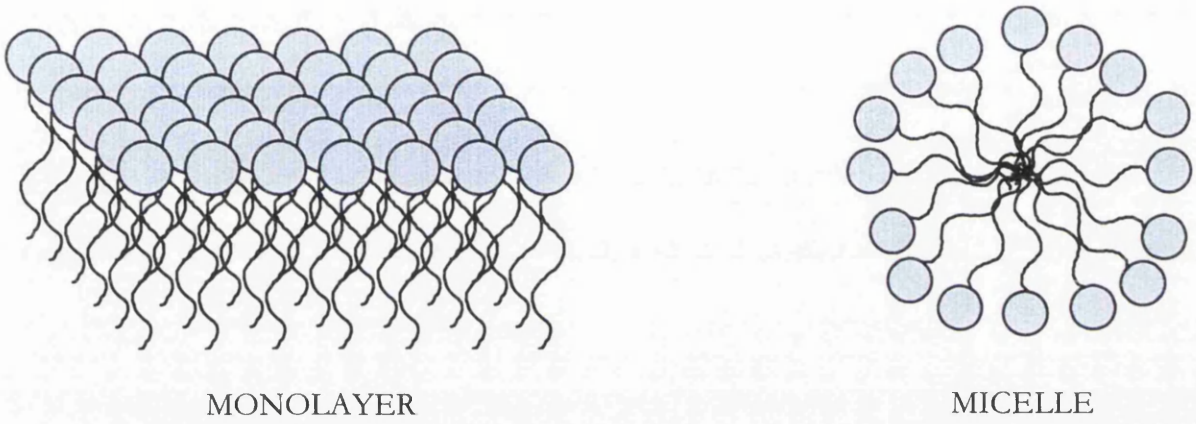


Figure 7: Comparison of Monolayer and Micelle assemblies of amphiphiles

Above a certain concentration, detergents solubilise through micelle formation, making up the second class known as soluble amphiphiles (Lichtenberg 1983).

A micelle is a self-assembling system in which the detergent molecules arrange themselves in a sphere so that the hydrophobic chains are in the centre and the head groups are on the outside.

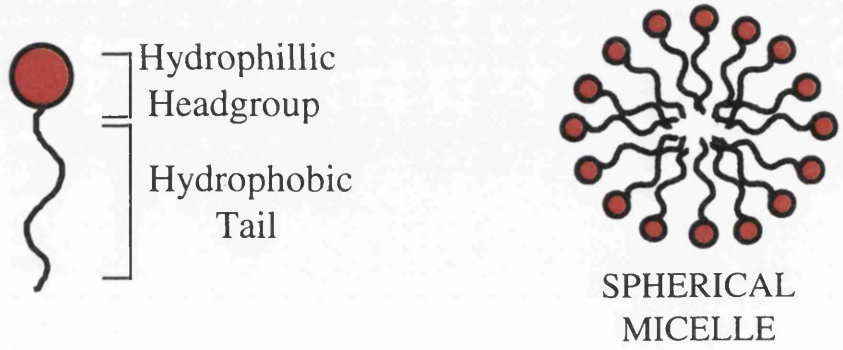


Figure 8: Diagram of a single detergent molecule and a spherical detergent micelle

This arrangement minimises the solvation energy, because the hydrophilic region remains wet while the hydrophobic region is shielded from water contacts. When a single detergent molecule is in aqueous solution the hydrocarbon tail is surrounded by an ordered structure of water molecules. When micelles are formed, water is expelled from the hydrocarbon region and replaced with hydrophobic interactions amongst the hydrocarbon tails. As the cage-like structure of water molecules around the hydrophobic tail breaks up, the entropy of the system increases and pushes the free energy of the system down (Neugebauer 1990). This occurrence, known as the hydrophobic effect, is not driven by the attraction between the hydrophobic tails but from the high energetic cost that would be required to dissolve the non-polar tail. In fact the hydrophobic reactions are quite weak, but their sheer abundance is enough to allow for spontaneous formation of these clusters. It is also important to note the energetic factors that oppose micelle formation. The electrostatic repulsion between head groups and the decrease in translational degrees of freedom may inhibit micelle formation. In addition, the solvation of polar head groups in certain solvents may have a high energetic cost. Hence, due to the hydrophobic effect, the formation of micelles is energetically favourable because perturbing the strong hydrogen-bonded water structure is too costly (Lichtenberg 1983).

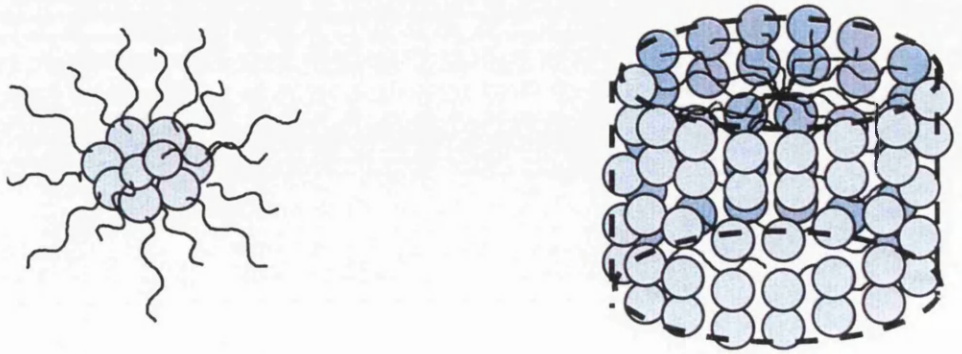
Considering the driving forces of micelle formation, one can imagine that certain characteristics of individual detergent molecule will affect their behaviour in solution. So the range in hydrophobic and hydrophilic regions invariably leads to a degree of variability in the type of micelle formed. Such changes in behaviour

can be predicted by a term called the packing parameter, P_p, which weighs the influential factors.

$$P_p = V_c / [a_o L]$$

Where V_c is the critical volume of the tail , a_o is the optimal surface area of the head group, and L is the critical length of the tail (Neugebauer 1990). This term is also useful because it helps us to understand how factors like salt, temperature, pressure, and pH changes can influence the micelle structure. For instance, consider a solution containing an ionic detergent. At high ionic strength, the ionic head group is surrounded by counter ions, allowing the them to pack more closely together. As the optimal surface area for the head group, $a_{o,}$, is lowered, the packing parameter increases. This accounts for the transition from spherical to cylindrical micelles in some ionic detergent as the ionic strength increases (Neugebauer 1990).

P_p	PREDICTED STRUCTURE
$< 1/3$	Spherical
$1/3 < P_p < 1/2$	Cylindrical
>1	Reverse



REVERSE MICELLE CYLINDRICAL MICELLE

Figure 9: Table and diagram of packing parameter predicted micelle structure

Similarly, the formation of reverse micelles that occurs in organic solvents can be predicted from the favourable interactions between the hydrophobic tail and the non-polar solvent (Scopes 1994).

Another governing factor in the formation of these micelles is detergent concentration. For example, at low concentrations there are not enough detergent molecules to form micelles. Hence the concentration at which there is enough detergent present to form micelles is called the *critical micelle concentration* or CMC. This value usually ranges from about 0.1mM to 30mM for the most common detergents (Neugebauer 1990). Most detergents have a narrow CMC, but some may have a range of values, depending on the method of determination (Lichtenberg 1983). There are two models used to describe the process of micellization at the CMC, the phase separation model and the mass action model. The phase separation model invokes the idea that at the CMC, phase separation occurs resulting in a detergent rich phase and the bulk solution. Above the CMC, the concentration of monomeric detergent in the bulk solution remains constant. On the other hand, the mass action model, assumes that the monomers and the micelles are in equilibrium. This means that the monomer concentration is no longer constant but equilibrium dependant. So above the CMC, monomer concentration may vary (Neugebauer 1990).

Whichever model is considered, another important characteristic of micelles is their aggregation number, N, or the number of detergent molecules in each micelle. Although this term may imply that every micelle contains the same

number of molecules, there can actually be a significant size distribution within a solution. So the N value actually represents an average value over a population. In fact, this value can also vary with experimental conditions. Certain experimental factors such as concentration, temperature, pH, salts, and additives can influence both the N and CMC values. For example, at high ionic strength head group repulsion is lowered so more molecules can pack in a micelle so the N value increases. Changing the size of the hydrophobic moiety also affects the aggregation number by altering the hydrophobic character of the detergent (Lichtenberg 1983). Certain ionic detergents seem to have a concentration dependant aggregation number (Neugebauer 1990). So as the concentration increases so does the size of the micelle. In addition to considering the CMC and aggregation number one must also pay attention to the cloud point of a given detergent. At the cloud point, the detergent shifts from a solution of isotropic micelles to a detergent rich phase and a detergent poor phase. When phase separation occurs, the solubilised membrane protein will partition into the detergent rich fraction (Ducruix and Geige 1992). Therefore, for crystallisation purposes, it is usually best to work between the CMC and the cloud point, so that there are micelles but no phase separation.

Now that we have an idea of the parameters that influence micelle formation, we can examine the actual structure. Assuming that we have a spherical or cylindrical structure, the micelle is composed of three major areas

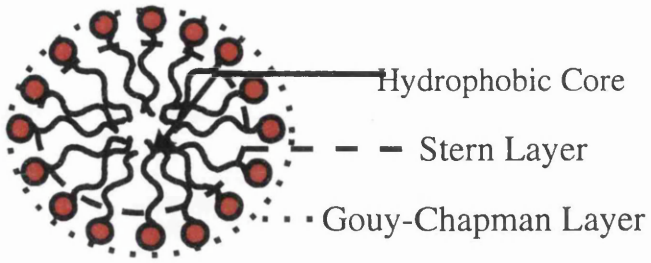


Figure 10: Diagram of designated micelle areas

The central core is composed of the hydrocarbon tail associated to form a liquid like hydrophobic centre. The surface is composed of the hydrophobic head groups along with the first layer of counter ions. This is known as the Stern layer and is usually about 1-3Å wide. Although it may seem like a sharp line between the hydrophobic and hydrophilic region, there is some overlap but in this simplified model we do not need to consider it. The final layer is the next 100Å adjacent to the Stern layer of solution containing bulk counter ions, known as the Gouy-Chapman layer (Lichtenberg 1983). Once this system of thermodynamically favourable isotropic micelles has formed the detergent molecules within the micelle can laterally diffuse through the surface. They are also in equilibrium with the monomers in solutions, resulting in some exchange. It is this micellular structure that makes detergents useful for protein crystallisation. In a given micelle, the hydrophobic surfaces of the protein make contacts with the hydrophobic core of the micelle and the hydrophilic portions can protrude into the polar solvent. It is this structure of the micelle that has made such an indispensable contribution to the world of crystallisation. Part of the difficulty of working with membrane proteins is their tendency to aggregate (McPherson 1982) This aggregation is driven by the hydrophobic effect. In an

effort to minimise energy of their hydrophobic surfaces, protein molecules will associate, leading to aggregation instead of crystallisation. When detergents are added to system, they "tie up" the hydrophobic surfaces and help to prevent such unfavourable aggregation events. Then the crystal contacts are free to form between the exposed hydrophilic regions of the protein surface. Hence, detergents greatly facilitate the crystallisation of membrane protein and therefore require a good understanding of their structures and behaviours if we are to use all their attributes to our advantage.

CHAPTER 10:

GRAMICIDIN BACKGROUND

Having examined the importance of membrane proteins and detergents, we are now ready to discuss the channel forming peptide antibiotic, gramicidin A. Gramicidin is the most well characterised of all the membrane channels. For this reason, it provides a simple model for larger, more complex membrane spanning channels. This fifteen residue peptide consists of alternating L and D amino acids (Sarges and Whitkop 1965):

(HCO)NH-Val(L)-Gly-Ala(L)-Leu(D)-Ala(L) ⇒

⇒Val(D)-Val(L)-Val(D)-Trp(L)-Leu(D)-⇒

⇒Trp(L)-Leu(D)-Trp(L)-Leu(D)-Trp(L)-

(C=O)NHCH$_2$CH$_2$OH

From the sequence alone, a number of factors that influence the function of gramicidin become evident. First, both the N and the C termini have been modified. The N terminus has been formylated while the C terminus has an ethanolamino group. In addition to the terminal modifications, the sequence is composed of alternating L and D amino acids. This alternation of sequence imposes certain constraints on the resulting structure. Due to the alternating L

and D amino acid sequence, gramicidin A adopts a β helical structure with a 6.3 Å diameter (Urry 1971). The resulting structure is known as the $\beta^{6.3}$ channel and forces the hydrophobic side chains to the outside of the channel. This leaves a 4 Å polar channel through the centre of the structure composed of the peptide backbone[*see INSERT figure 11*]. Another important feature of the sequence occurs at position 11 of the amino acid sequence [highlighted in blue]. Gramicidin is produced by the bacteria *Bacillus brevis* in three naturally occurring isoforms (Hotchkiss and Dubos 1940). These isoforms vary between tryptophan, phenylalanine, and tyrosine at position 11 of the sequence

GRAMICIDIN ISOFORM	POSITION 11	NATURAL OCCURRENCE
A	Trp	72%
B	Phe	9%
C	Tyr	19%

Figure 12: Table of gramicidin isoforms and their natural occurence

From the above chart (Stankovic 1990), we can see that gramicidin A is the most plentiful and is therefore the most commonly studied. Until recently, purified gramicidin A was not available and therefore required the use of a flash chromatography system to purify gramicidin A from the other isoforms (Stankovic 1990). This method is very time consuming and yields are less than fifty percent.

Having examined the properties of the primary sequence, let us now examine the functions of this peptide. As a dimer, gramicidin makes the

membrane selectively permeable to monovalent cations, such as sodium, lithium, cesium, rubidium, and even ammonium (Busath 1993). These monovalent alkali metals make contacts with the oxygens of the carbonyl groups on the peptide backbone that lines the channel. Multi-valent cations cannot traverse the channel due to their large solvation shell and anions cannot make the proper contacts with the carbonyls to get into the channel, resulting in a strong selectivity. Due to these characteristics, gramicidin has been widely studied for its conducting properties (Eiseman 1983). Many factors, including both side chain and covalent modifications have been altered to check its affect on conductance (Barret 1986). In addition the choice of phospholipid also has an effect because certain phospholipids form a thicker bilayer (Neher 1977).

In order to understand how all these factors affect the function of gramicidin we must examine the proposed structure of the dimer. As mentioned earlier, in a bilayer gramicidin adopts a β helix with 6.3 amino acids per circumference (Urry 1971). Although Urry originally proposed a left-handed helix, NMR experiments have shown the channel to be right handed (Arseniev 1990) and this has been further confirmed by bilayer studies (Chui 1991). The monomer is not long enough to span the membrane, but when another monomer on the other side of the bilayer randomly diffuses into the proper orientation, six hydrogen bonds form between the N termini of the monomers. These intermolecular hydrogens bonds resemble those of an antiparallel β sheet and stabilise the conducting species. This interaction produces a dimer [*see INSERT,*

figure 13] approximately 25 Å in length (Busath 1993). This distance is just long enough to span the lipid bilayer with only a slight puckering effect.

The channel itself is approximately 4 Å wide, leaving room for a single line of water molecules. One might guess such a thin aqueous channel would not be very effective for ion transport, but this is not the case. In fact, 10^7 sodium ions can pass through one gramicidin channel in a single second (Stryer 1975). This value is only an order of magnitude less than diffusion in pure water. With such a high rate of diffusion, one must wonder how the diffusion of cations is controlled. Since the monomer cannot conduct ions, the formations of the dimer is the limiting factor. Within the membrane, the monomer is in equilibrium with the dimer. Hence, dimerisation is a random event which depends on the random lateral diffusion of the monomer and governs the movement of ions.

In order to understand how modification of this peptide affects its function there has been a great drive to elucidate the structure of the gramicidin channel. In addition to the single-stranded, right-handed head-to-head channel form, gramicidin also has been found to arrange dimers in a "pore" conformation (Veatch 1974). This alternate structure is composed of two intertwined antiparallel gramicidin monomers, connected by 28 intermolecular hydrogens bonds (Wallace 1988). These structures vary in internal radius from 5.6 (Langs 1988) to 6.4 Å (Wallace 1988) and have been shown to have two cation binding sites 20Å from each other near the openings of the pore (Smart 1993). Unfortunately the pore has proved to be a hindrance to the structure solution of the channel because in organic solvents or lipid with unsaturated fatty acids tails,

gramicidin adopts this pore structure. In fact, all of the crystal structures (Langs, 1988, 1991) (Wallace 1988) to date, have described the pore conformation and not the channel. Herein lies the classic complaint concerning crystallographic structures. Although, one may determine the three dimensional structure of a molecule like gramicidin, it is only a static representation and it may not be the active form of the compound. This is exactly the case for gramicidin. The structure was solved for the pore conformation, but crystals of the channel conformations have proved more elusive. It may be argued that the structures of the two conformers might somehow be related since interconversion must occur between the two. This would mean that structural information for the channel might somehow be inferred from the pore structure since gramicidin must interconvert between these two dimers. Despite the uncertainty surrounding the mechanism of insertion into the bilayer, one theory involves a transition from the pore conformation to the channel (O'Connell 1990) According to one of O'Connell's theories, gramicidin adopts the pore conformation in bulk solution, attaches to the membrane surface and then inserts itself. After insertion, the intertwined pore structure is proposed to unwind and form the head to head channel. This theory would provide a good model where the structure of the pore conformation might have an influence on the structure of the channel. Unfortunately, I think this mechanism of insertion is unlikely since the pore is composed of left-handed helices whereas the channel is composed of right handed helices. This would require unwinding of the two anti parallel helices and a simultaneous switching of handedness. After such a large conformational change,

it may be difficult to infer commonalities between the structures with any degree of accuracy.

CHAPTER 11:

APPROACH

After examining some of the literature on gramicidin, it seems the missing link in much of the literature is a crystal structure of a channel conformation. The major goal of this project was to try and overcome the obstacles of growing gramicidin crystals by adopting a new two fold approach. A recurring problem in the crystallisation of gramicidin it that the channel dimer seems to convert to the pore conformation when supersaturated. As an undergraduate, I worked with Dr David Crumrine at Loyola University Chicago and Edward Quigley at Stritch Medical School to help covalently modify gramicidin with various linking groups to lock the channel in an open or closed conformation. I worked on the synthetic end of this project. Although the main goal of the project was to synthesise these compounds for conductance and gating studies, they also seemed ideal for crystallisation studies. If the dimer is covalently linked in a head to head fashion, then it should not be able to adopt the pore conformation without breaking these covalent bonds. This should eliminate the problem of interconversion to the pore. The second component of my approach is to treat gramicidin as a membrane protein rather than a small molecule. Due to gramicidin's small size[only 15 residues], it makes sense to treat it like other small molecules. For this reason, standard small molecule techniques such as organic solvents, were used to induce supersaturation and produce crystals of the pore conformation (Lang 1988,1991) (Wallace 1988). Instead, I wanted to attack the crystallisation problem from the

standpoint that gramicidin is a small membrane protein. This would mean using use membrane techniques such as detergents to try and mimic the hydrophobic portion of the membrane. Since the channel is the preferred structure in the membrane, if the hydrophobic interactions of the membrane could be successfully replaced with micelles interactions, the channel form should be favoured. Between these two approaches, the covalent linkage and the use of membrane techniques, the likelihood of channel formation over pore formations should increase and thereby increase the chances of crystallisation of the channel dimer of gramicidin.

CHAPTER 12:

TECHNIQUES

Since the covalently modified dimers of gramicidin had previously been prepared, I began my work by focusing on mimicking the membranous environment of native gramicidin. I had about 1 gram of purified gramicidin A and less than half a gram of dioxilane linked gramicidin. Having limited quantities of the linked dimer I decided to use the pure gramicidin A as trial compound and then any method or conditions that seemed promising would merit use of the linked dimer. In order to become familiar with the techniques for crystallisation, I began setting up crystallisation trials for Hen Egg White Lysosyme[HEWL]. Due to its ease of crystallisation, HEWL is regularly studied and known to crystallise from 8 to 10 %(w/v) sodium chloride solution of a 2mg/ml HEWL at a pH of 4.5 (Ducruix and Geige 1992). Varying both pH and salt concentration around these conditions, I set up 24 independent vapour-diffusion experiments in a single crystallisation tray. This is known as a *grid screen* because two parameters can be varied at the same time. It also allows for the optimisations of parameters. In this case, the concentration of sodium chloride ranged from six, eight, ten, and twelve percent weight per volume, while the pH ranged from 4.2, 4.5, and 4.8 using sodium acetate buffer. Since vapour diffusion was the main method I used to screen crystallisation parameter it deserves a closer examination. As the name implies, this type of experiment use vapour diffusion to bring a protein solution to a state of supersaturation. A normal experiment consists of a well containing the

drop of protein solution suspended over a relatively larger volume of reservoir solution. The reservoir contains a higher concentration of the precipitant than the well solution. Once the unit has been sealed with crystallisation tape, this difference in concentrations will drive the system to equilibrate. Since the reservoir is more concentrated, water will migrate from the drop to the well, until the concentration of precipitant is equal in both solutions. The goal of this is to bring the solution to the appropriate level of supersaturation to induce crystallisation. Although this type of experiment can be done on macroscale volumes, I used the sitting drop method which typically uses a one millilitre reservoir and two to twenty microlitre drops. This has the added advantage of using very small amounts of protein and allows for a great number of experiment to run simultaneously.

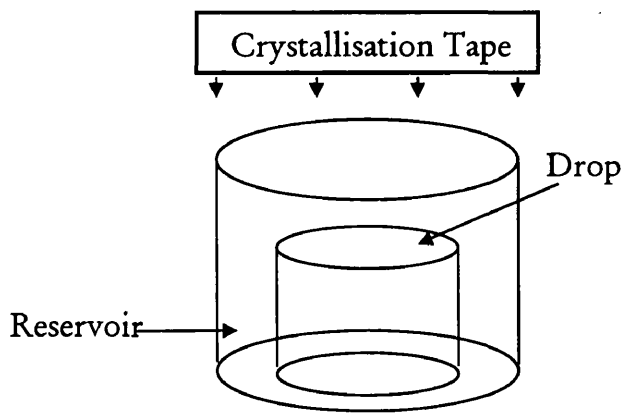


Figure 14: Diagram of a sitting drop vapour diffusion experiment

In the sitting drop method shown above, the drop of protein solution rests on a pedestal above the reservoir. Another common variation of the vapour diffusion experiment is the hanging drop method. In this case, the crystallisation

tape is replaced with a siliconised glass slide and the drop is suspended from the slide. This means there is no need for a pedestal. The hanging drop method is extremely useful when crystals tend to stick to the bottom of the well. When crystals form in hanging drops, gravity will pull them to bottom of the drop. Unlike sitting drops, there is no surface for the settling crystals to adhere to. Incidentally, hanging drops are more dependant on the surface tension of the drop. Hence, hanging drop experiments are limited to solvents and compound that maintain this requirement. This rules out highly volatile solvents or detergents that would disrupt the surface tension. Due to these limitations, I utilised sitting drop vapour diffusion as my method for bulk screening experiments.

After crystals of lysozyme had formed, I went through the process of mounting and collecting data. These are skills that can only be learned by experience.

Procedure For Capillary Tube Crystal Mounting

1.) Remove small capillary tube with forceps and hold near the narrower of the two ends, making sure to leave enough room to cut off the end. Then, draw the glass cutter once along the thin end of the tube and carefully snap off the sealed tip to produce an even end. Now, attach the tube with syringe or mouth piece to the wide end of the capillary tube.

2.) Razor open the well of choice and draw up a few millimetres of solution. While looking through the microscope glass, insert the glass tube into the well and carefully pull up the crystal. If the crystal is stuck it can be gently

dislodged with an animal whisker. These usually work without cracking the crystal.

3.) Remove the crystallisation tray and reduce the magnification so that you can see most of the tube and the crystal. Use a paper wick to remove the mother liquor from around the crystal. As you get nearer to the crystal, make sure to rotate the tube so that all the liquid adjacent to the crystal is removed. Ideally, you would like to remove all the liquid in front of and adjacent to the crystal, while leaving some solvent behind and making sure not to damage the crystal. If you accidentally remove all the solvent, just pull up some more well solution, taking care not to wet the crystal

4.) Now the narrow end of the capillary is sealed with beeswax and the tubing removed. Now the wide end of the capillary is removed with the glass cutter to produce a capillary tube of uniform width. This end is also sealed with beeswax and then mounted in a sphere of plasticene.

This mounted crystal was then place on a goniometer head and centred in front of the image plate detector. There is an attached microscope that enables you to view the crystal while it is rotating. You must centre the crystal properly to ensure that the crystal is always in the beam. To double check the alignment, it is best to take to exposure at 0° and 90°. After these frames have been recorded, you can check the diffraction pattern for intensity, background, and twinning. If there is no twinning and the peaks are resolvable from the background, you can

set up the diffractometer to collect the data set. In this case, each frame took ten minutes to collect and the complete set was collected overnight.

In addition, to this room temperature data collection, data was also collected 100 degrees Kelvin. Cryocooled crystals tend to last longer than crystals at room temperature (Ducruix and Geige 1992). Presumably the lower temperature reduces the amount of free radical formation that often shortens the lifetime of a crystal. In many cases, under cryocooled conditions a single crystal can provide a complete data set. Another added benefit is that cryocooling reduces the amount of thermal motion of the molecules in the crystals. With less net movement of molecules, cryocooled conditions can provide better resolved structures. The procedure for cryocooling is slightly different from room temperature. The crystal is not mounted in a capillary tube, but suspended in a loop of cryoprotectant. This cryoprotectant is usually composed of the well solution and between ten and thirty percent glycerol.

Cryocooling Procedures

1.) On a glass slide pipette a 20 microlitre drop of cryoprotectant. Using a loop only slightly larger than the largest dimension of the crystal size, carefully pass the loop through the cryoprotectant and place it immediately into the cryostream. Speed is of the essence at this step. Outside the cryostream is a dry stream so that no atmospheric water can get into the cryostream. If you leave the cryoloop in the atmosphere too long it will pick up water. This will result in ice crystals in the loop which will make it appear cloudy. The ice crystals will distort the diffraction pattern of the protein crystal, resulting in ice rings.

For this reason is it necessary to optimise the cryoprotectant by adding additional glycerol until the loop appears clear. This must be done *prior* to looping the crystal. Place a fresh drop of cryoprotectant on the slide and keep it in close proximity to the microscope and cryostream.

2.) A. The crystallisation tray is place on the microscope and the well is razored open. The loop carefully drawn through the well to scoop up a crystal. As soon as the crystal is looped, it is then transferred to the drop of fresh cryoprotectant on the slide. It is immediately re-looped and place in the cryostream. The loop should look clear except for the crystal.

B. An alternate method: Instead of transferring the crystal, sequentially inject small portions of the cryoprotectant to the drop. Then remove an equal volume from the drop. When this process is done a few times, it will exchange drop solution for cryoprotectant. Then the crystal can be looped and mounted directly into the cryostream with no transfer step.

Despite the choice of method, the exchange of solvent for cryoprotectant can often cause cracking, by disrupting the solvent molecules in the crystal. For this reason, using the least amount of glycerol is optimal. The less the mother liquor changes, the less trauma for the crystal and the less likely it is to crack. In some cases, the mother liquor alone is sufficient as a cryoprotectant. This is often the case, especially for solutions that contain polyethylene glycol. Using both room temperature and cryocooled conditions, the data was processed, refined, scaled and

fit, but I will not discuss this process in detail because lysozyme has been solved many times. It was for my own educational benefit to familiarise myself with the techniques and software.

CHAPTER 13:

CO-CRYSTALLISTION

Having acquired some of the basic skills to proceed, I focused on gramicidin. In 1991 Wallace published an article on the co-crystallisation of gramicidin and phospholipid. These crystals diffract to 2 Å resolution, belong to the space group $P222_1$ and have a unit cell containing one gramicidin monomer and two phospholipid molecules (Wallace 1991). They propose that the lipids take up a bilayer pattern and the gramicidin monomers make end-to-end contacts. This type of formation seemed an optimal system to replace the membrane with phospholipids and then crystallise the entire system. It also had the added advantage that I could use the same phospholipids that had been used for the conductance and gating studies and hopefully compare the results. To become familiar with the procedure, I decided to set up comparable crystallisations. This step had two major problem. First, the crystal described in this article took "at least six months (Wallace 1991)" to grow. Obviously focusing my work on a technique that would take at least half of my allotted time to develop any results is not a very appropriate method. Secondly, the details in the article for the method of crystallisation were vague. For example, when referring to the setting up of crystallisation trials, Wallace writes, "to *some* samples, deionized water was added (1991)." It was unclear, whether these were the conditions that produced crystals or the ones that did not. Below is a segment of the procedure portion of the paper:

> Crystals were prepared as follows: 50.0mg of gramicidin A and 50 mg of dipalmitoyl phosphatidylcholine were dissolved in 1ml of absolute ethanol and heated to 55° C for ten minutes. To some samples, deionized water was added to make a 2% solution(v/v) and the specimen left to crytallize at 20° C in the dark by very slow evaporation, over a period of six months with minimal disturbances (Wallace 1991).

Since I had a limited supply of gramicidin, I scaled down the solution to 15 mgs of each, but I consistently had the same solubility problem. Whenever I diluted with deionised water, the protein and phospholipid instantly precipitated out of solution. Initially, I suspected that I had just let my ethanol solution of gramicidin A and DPPC cool down and then it was no longer soluble in the water, but closer inspections showed this was not the case. I even tried making the 2% dilution with warmed water solution to see if it was a function of the temperature. It was only soluble between 50°C and 55°C and as soon as the solution cooled, it would become cloudy. Despite my efforts to keep it from precipitating, this 2% dilution consistently crashed out of solution. At this point, I contacted Wallace and asked her to clarify some of the procedural points. Although she did not directly answer my specific queries, she did suggest that I come to her laboratory and talk to her in person. Unfortunately when I contacted her to set up a time to visit, she never responded. At this point, I decided not to use her procedure since the gramicidin and phospholipid were not soluble in the 2% ethanol/water mixture.

Instead, I made up protein solutions with phospholipid in ethanol instead of water. In the presence of organic solvent and phospholipid is difficult to predict whether gramicidin will take up the channel or the pore conformation, but my plan was to equilibrate this solution against the water based screening conditions. The introduction of water should push the equilibrium to the channel and, the drop should pass through the desired level of supersaturation at some point during the equilibration. I screened this solution through the Magic 50, Magic 50B, and the Footprint solubility conditions, but almost every condition resulted in amorphous flocculating precipitant. The only promising condition was well 40 of the Magic 50 B conditions consisting of 25% tert-butanol, .1M Tris buffer pH 8.5 and .1M $CaCl_2$. In my excitement, I left the light on when I ran to have someone inspect the crystals and the crystals melted from the light of the microscope. This tray had been jostled prior to equilibration and some of solution splashed around so it was impossible to know if any thing else had contaminated the well. Regardless of these two misfortunes, I tried to optimise these conditions with a number of grid screens varying the pH, calcium chloride concentration and the percentage of tert-butanol. The only crystals that developed were very small and turned out to be tert-butanol.

Since none of the screens seemed to be very successful, I altered the composition of my protein solutions. Previously, the precipitate under most conditions was almost chunky and globular in nature. This seems to suggest high level of aggregation. To combat this problem, glycerol and the detergent LDAO were added to the solution. Hopefully, the detergent would form micelles around

the channel and the glycerol would perturb the water structure and influence the solubility of the phospholipid (Sousa 1990). The resulting solutions contained between 5 and 10 % glycerol and between 0.1 and .5% LDAO[N,N-dimethyldodecylamin-N-oxide]. These alterations had a dramatic effect on the amount of water that could be introduced. This meant I could replace pure ethanol with an azeotropic solvent system of glycerol, water and ethanol.

Figure 15: Composition of solutions 1 - 4

1mL solutions	Solution 1	Solution 2	Solution 3	Solution 4
Gramicidin(mg)	2.7	2.7	2.3	2.3
DPPC(mg)	13.4	13.4	11.5	11.5
Glycerol	3%	3%	4%	–
Water	20%	20%	33%	57%
Ethanol	77%	77%	63%	41%
LDAO	.3%	.5%	.5%	.5%

The working solutions were mostly 1 and 2, solution 3 would often precipitated overnight for no apparent reason and solution 4 was to check the necessity of glycerol. Since, I was unsure of the full solubility effects this would have on my working solutions, I decided to run small scale solubility tests against various salts. Under a microscope, a 10 to 15 microlitre drop of protein solution was observed while .5 microlitre was sequentially added until precipitation occurred. This was originally investigated with both 1M calcium chloride and 1M ammonium acetate, but there was little consistency to the results. For example, a 10 microlitre drop could require anywhere from .2 to 1.2 microlitres of acetate to form precipitate. If the protein precipitates at different concentrations of salt, then

the salt cannot be sole reason the precipitation. This lead me back to the question of the water threshold. Instead of using, salt as a precipitant , I used water and obtained the following results:

Figure 16: Percentage of water necessary to induce precipitation in various solutions

Solution	1	2	3	4
Water	34-38%	28-32%	33%	61-68%

These results show that glycerol was not a necessary component of the system. In fact, when glycerol is removed the solution can accept almost twice the amount of water before precipitation. It also elucidates the crucial role of water in these experiments. Although this may seem trivial, it is very important because it exemplifies the point that in the some systems, water can act as a precipitant. For most protein crystallisations, we usually think of salts, polyethyleneglycols[PEGs] and organic solvents as the precipitant (McPherson 1982), but now the roles are reversed. Contrary to most systems, water is not the solvent, but the precipitant.

Which brings me to my next point, the problems of such a system. One might ask why try to change the solvent system if you know what makes it precipitate? Firstly, when gramicidin A is in organic solvents it adopts a pore structure which is not the desired form to crystallise. In order to secure the channel form, I need to change the solvent system to one with as little ethanol as possible. One way to achieve this is to remove glycerol, as shown by the solubility experiment. The next problem of organic solvents is their volatility. In a vapour diffusion experiment, a slow equilibration often results in more well-ordered crystals. This problem is well addressed for an aqueous system, but not

for the case of an azeotropic system. For example, in a system of water and ethanol, both components can vapour diffuse. This cross-diffusion leads to a quicker equilibration time and can promote precipitation over crystallisation. Another problem due to the volatile nature of the solvent was evaporation of the drop while setting up the crystallisation trays. The ethanol azeotrope evaporates very quickly. This alters the composition of the solution and thereby alters the solubility of the protein. Evaporation can change the volume of the drop significantly and introduce another variable into the experiment, especially when small volume drops are concerned. Considering all these factors, it is clear why changing the solvent system might have a number of benefits.

CHAPTER 14:

SOLUBILITY

In addition to the macroscale solubility experiments, I carried out another solubility experiment to determine the water threshold of solutions. I equilibrated 5 microlitre drops of solutions 1-4 against various ethanol water mixtures. The ratios of water to ethanol ranged from 5% to 60%. The point where precipitation starts would provide me with a narrower range of the water concentration necessary to induce supersaturation. These results confirmed the results of the macroscale experiments. In addition, for solution 2 small microcrystals formed in the wells containing 25-30% water after about two weeks. These crystals form rectangles with dimensions 5 by 10-15 microns and were readily reproducible. According to the solubility diagram, a shower of microcrystals is indicative that crystallisation is occurring too close to the border between precipitation and nucleation[See X on the diagram below].

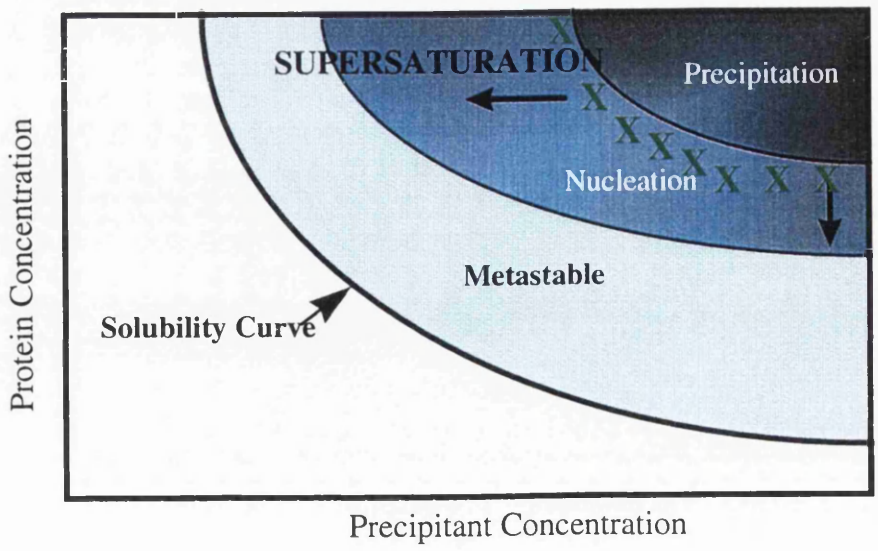


Figure 17: Phase diagram of solubility (Ducruix and Geige 1992)

There are three major approaches to this problem. First, reducing the amount of precipitant should move back across[←] the nucleation area and away from precipitation zone. I had covered this approach during the solubility test and it did not change the microcrystalline nature of the precipitant. The second approach would be to lower the protein concentration and move down [↓] away from the precipitation region. Since solution 2 had a protein concentration of 2.7 mg/ml of gramicidin, similar experiments were carried out at one, two, three and four times dilution. Unfortunately, this alteration did not produce any larger single crystals. In the wells that had positive results, the microcrystals were the same size or smaller and were not as well ordered. The third method I used was to try and slow down the process of equilibration. This was done in a number of ways. First, I tried setting up the crystallisations in the cold room at 4°C. This only lower the solubility of the gramicidin, causing precipitation within half an hour at very low percentages of water, even 5%. Other temperatures, between the cold room and 22°[crystallisation room temperature] were also set up in the incubator but caused the same tendency in precipitation as the 4°C results.

In an attempt to grow larger, more suitable crystals for diffraction, I began exploring other methods, such as seeding and crystallisation in gels. The technique of seeding has become a regular technique in crystallogenesis and is therefore addressed more thoroughly in the first half of this discussion. In short the technique requires the introduction of seed crystals to a pre-equilibrated system. The idea being that the solution only needs to reach the metastable region

to begin crystal growth on the seeds. Such a system avoids the pitfalls associated with the nucleation region of solubility. I used the streak seeding method to determine the optimal well solution [40-45% water] and protein concentration [1.5 -0.75 mg/ml]. I could not use the macroseeding technique to deliver a nucleation site, because there was no way to pick up a single crystal. Instead, I employed a system of multiple dilutions which worked nicely since the microcrystals were small enough to not require any further crushing. Unfortunately, the only crystals that resulted were still microcrystalline in nature.

CHAPTER 15:

SILICA GELS

The second approach was to use silica gel as the media for equilibration instead of vapour. The movement of solvents are greatly hindered when traversing gel media rather than the atmosphere. This factor slows down the entire equilibration process. Two methods of gel crystallisation best suited to my work were tube crystallisation and one adapted to the sitting well trays (Ducruix 1992). In the first method, silica gel is pipetted into the middle a tube of variable length but small diameter, approximately half a centimetre or less. When the gel solidifies it will form a wall which separates the tube into two compartments. The protein solution is placed on one side of the gel wall and the other side is filled with 'reservoir' solution. The two ends of the tube are then sealed and the system left to equilibrate. This method was used to test 25-40% water solution and 0.75 to 3.0 mg/ml, but none of these produced anything crystalline, only precipitation or nothing at all. Since, this system did not work, I tried another method adapted for sitting drop vapour diffusion. In this system, a 20 microlitre drop of gel is delivered to the well and allowed to solidify. Then 15 microliter of the protein solution is pipetted onto the gel and allowed to adsorb. This process can take up to ten minutes. Due to the volatile nature of ethanol, this method proved somewhat awkward. In all my sitting drop experiments, I would first pipette the water portion of the reservoir, followed by the ethanol portion of the reservoir and finally, the drop itself. I tried to minimise the evaporation component by

sealing the wells as soon as possible. But when using the gels, the ethanol seemed to evaporate much more quickly, often resulting in visible precipitant before the tray has even been sealed. Possibly the water portion of the azeotrope adsorbs to the gel first and leaves the ethanol to evaporate more rapidly from the large surface area of gel. Another complication arose after the trays had been left to equilibrate. Instead of remaining solid, the gel had dissolved and re-liquified over the equilibration period. Undoubtedly this is a result of one of the components of my protein solution, but which one I could not specifically determine.

CHAPTER 16:

REMOVAL OF DPPC

Once again, this recurring problem of the solvent system has reared its ugly head. In an attempt to resolve this dilemma, I had to address two issues, the current azeotrope and the possibility of an alternate solvent. In order to determine if any of the components of the azeotrope were dispensable, I formulated different protein solutions to vary the combination of component. The component of the solution were gramicidin, DPPC, glycerol, LDAO, ethanol, and water. Since water and ethanol were technically the solvents, I decided to focus on the additives, glycerol, DPPC and LDAO. From the microscale solubility experiments, glycerol had already become suspect as an efficient additive. Each solution was composed of about 2.7-3.2 mg/ml gramicidin and then one other additive. This solution was set up to equilibrate against a range of 5-60% water solutions, and the crystallisation results are listed below:

Figure 18: Table of crystallisation conditions involving DPPC

Solution	GramicidinA	DPPC	Glycerol	LDAO	**RESULTS**
1,2	⊗	⊗	⊗	⊗	Microcrystals
3b	⊗	--	--	⊗	Dust-like precipitant
4b	⊗	--	⊗	--	Spherical precipitant
5	⊗	⊗	--	--	Microcrystals
6	--	⊗	--	--	Microcrystals

where ⊗ represents presence in the solution

From these results, it was clear that the microcrystalline product for which I had been optimising conditions was the phospholipid, DPPC and *not* the

intended target, gramicidin. This discovery, albeit frustrating, led to a very important shift in my work. I began a literature search to see if crystallisation of phopsholipids, like DPPC, was a common occurrence and how it might be resolved. What I found was that no one uses phospholipids for membrane crystallisation, apart from the one example I had started with. In fact, people go to great lengths to eliminate them from there membrane proteins because they lead to disorder during the crystallisation process. In most cases, detergents were used to solubilise membrane protein and eliminate the phospholipid entirely (Neugebauer 1990). At this point, I decide to completely eliminate phospholipid from my protocol for gramicidin A and replace it with a detergent.

CHAPTER 17

SOLVENT

Despite this major change in the approach to this project, the immediate problem was still the solvent system. Ideally, I was looking for a non-volatile solvent that would keep the gramicidin soluble enough to set up sitting drop experiments. Originally, I had set up the following experiment in methanol to assess the role of the detergent, and determine the solubility of gramicidin alone versus the presence of detergent. Although methanol was far from the best candidate it surprisingly produced crystals. A 2.9 mg/ml solution of gramicidin was equilibrated against 5-60% water(in methanol) solutions and found to precipitate out at higher than 40% water. Then detergent was added to make a 0.5% LDAO solution and equilibrate against the same conditions. Initially, the wells above 35% formed precipitant accompanied with phase separation above 55%, but after about five weeks crystals had grown on a fibre in the 45% well and later in the 35, 40 and 50% wells, crystals also developed. This observation led to the use of ~3mg/ml gramicidin, .5% LDAO in methanol as a standard solution to test other solvent system.

With a new standard system, other solvents such as isopropanol, 2,5 hexanediol, and 2,methyl-2,4,pentanediol(MPD) were readily evaluated. These solvents are also favourable because they are less harsh than other organic solvents. Solutions of 3mg/ml gramicidin and .5% LDAO were made for each of these solvents and then equilibrated against various percentages of water. Both

isopropanol and hexanediol only produced chunky flocculating precipitate, whereas the MPD produced dust-like precipitate. The chunky precipitate usually indicates a sudden shift in solubility. On the other hand, the dust-like precipitate of protein under MPD conditions may indicate a slower equilibration. This hypothesis was confirmed as the MPD conditions took about two weeks to form precipitate, while both propanol and hexane form precipitate in a matter of days. For this reason, I focused on the range 50-80% water where precipitation occurred. I particularly liked the MPD system since it was slightly viscous which meant that evaporation was not such a large problem. This viscosity also eliminated the problem of cross-diffusion since MPD is not volatile. Another added advantage was that the water solubility range had shifted from the 30-40% water for ethanol and methanol to 50-80% water. Due to the benefits of the solvent methanol, this range was tested for a number of parameters, including pH. I examined a pH range of 4-8, altering the concentration of buffer between 50-100 mM with no success. I also tried a number of salts, including magnesium acetate, lithium sulphate, calcium chloride, ammonium acetate, manganese chloride and cobalt chloride, but they only produced precipitate or salt crystals. Phase separation was a common problem for many of the MPD conditions. This is particularly unfavourable because the protein will partition into the MPD rich bubble, while the salt will partition into the water portion. With no mixing supersaturation will never be reached. Unfortunately, none of the MPD conditions produced anything remotely crystalline.

CHAPTER 18:

AZEOTROPE METHOD

While working on the MPD system, I was also trying optimise the detergent/methanol conditions and grow larger crystals. Having previously determined the solubility range of the methanol solution containing 3 mg/ml gramicidin and .5% LDAO, it seemed appropriate to start examining other parameters such as salts, metals, PEGs, and pH. Although there are plentiful tools to quickly and efficiently screen a large number of the precipitants for soluble proteins, there is not such a large scale method for membrane proteins. For example, in the Glasgow laboratory, there are a number of solubility screens[M, N, Magic 50, Magic50B, Footprint solubility, and PEG 6K] which easily provide over 400 independent conditions. The challenge was to find a way to adapt these screens to a protein that is not soluble in water. This was achieved through the azeotrope method which requires that each reservoir solution is composed of both methanol and the given screening condition. Since the presence of new precipitant might change the solubility range, this must be accounted for by examining a range of azeotropes for each condition. For the case of gramicidin, I investigated the range of 20-50% water. Although this is not an exact system, it did provide a relatively quick method to highlight precipitants that warranted further investigation. Using this procedure as a screening process, certain conditions would result in shiny precipitate, "blobs" that resembled melted crystals or require a high level of water. In particular, low molecular weight PEG solutions required

more than 50% water to cause precipitation, and remained clear for the initial screens. For PEG 200, 400, 550, and 1000, I investigated varying percentages of PEG and lowered the percentage of methanol to zero in some cases. For most of these experiments, no precipitate developed or phase separation occurred.

After the PEG conditions, I examined the role of various metal ions including cadmium, iron, cobalt, cesium, zinc, lithium, and nickel. Since gramicidin can conduct monovalent cations, the introduction of some of these ions might help stabilise it and somehow affect the solubility. There is also a flipside to this argument. Metals like cesium are known to bind the pore conformation of gramicidin (Wallace 1988). Binding the pore conformation may shift the equilibrium or stabilise this unfavourable conformation. Unfortunately, the introduction of metals did not produce any promising results.

Acidity and pH was the next important parameter that was addressed. Since proteins are known to be least soluble at their pI, this can prove to be a difficult pH area to work around. In order to determine the pI of gramicidin, I ran an iso-electric focusing[IEF] gel. This method separates compounds based on the pI by running a current across a gel containing the compounds, the compounds will migrate to the cathode or anode depending on its overall charge (Stryer 1975). Then the gel is fixed, stained and the compound in question is compared to an internal standard. From the standard, you can ascertain the pI of a given compound. I ran a number of IEF gels, but had some minor complications. First, a number of the solutions used during the coomassie blue staining process contained methanol, so these steps washed the gramicidin right off the gel. So I

made up alternate solutions that did not contain any methanol, but it appeared as though the gramicidin never moved. This would mean that either the pI of gramicidin was exactly neutral, or that it was not moving along the gel. This lack of movement was confirmed by another IEF gel stained by an alternate method, silver staining. Despite the usefulness of IEF gels for most proteins, in the case of gramicidin this method proved inconclusive. Instead, I used one of the many programs available that determines the pI from the sequence of the protein. From the ExPASy web site, the pI of gramicidin was found to be 5.49-5.52.(PI site 1997). Since the pI was approximately 5.5 I decided to carry out an intensive set of experiments to examine the affect of pH.. Using Tris buffer at high pH, gramicidin was variably soluble in the range of 60-90% water, but nothing promising developed only precipitation. Similarly, there was nothing promising in any of the lower ranges either. Initially, citrate buffer was used to probe the 3.2-5.6 range but consistently formed buffer crystals. Acetate over approximately the same range 3.5-5.0, had less of a tendency to form crystals but only produced precipitate. Although I had originally intended to avoid the pI region, it was probed with MES buffer in a range of 5.0- 6.3 for the sake of completeness. In short, for the range of pHs I examined and the buffers chosen, none of them produced optimal conditions of supersaturation necessary for crystallisation.

CHAPTER 19

SCREENS AND OTHER METHODS

Another method for evaluating pH is through the low ionic strength screen. This method was originally developed for monoclonal antibodies, but is an effective method for specifically testing the solubility effects due to pH and temperature (Harris 1995). The protocol for the low ionic screen is different than normal sitting drop experiments. First, the drop is 11 microlitres in size, composed of a 4:2:5 ratio of protein: buffer: precipitant solutions. The buffers range in pH form 2-12, providing a wider range than normally available. The concentration of the buffer in the drop is prepared to be 10mM. The precipitant is PEG 3350 and ranges in concentration from 4-24% (w/v) (Harris 1995). Following the above protocol, I set up low ionic strength screens at three different temperature 4°, 12°, and 22°C all with no success.

In addition to the low ionic screen, I also tried the additive screen marketed by Hampton Research. Since many seemingly strange additives have proven to facilitate the crystallisation process (Ducruix and Geige 1992), it is often worthwhile to at least see if they have any effect on a given protein. Compound such as amphiphiles, chaotropes, chelators, polyamines, linkers, and reducing agents make up the screen of 48 additives. Despite its compatibility with membrane methods such as detergents, it produced no noticeable change in the solubility range of gramicidin.

After a presentation introducing the new membrane method known as lipidic cubic phases, I decided the system was well suited to gramicidin. The idea is that some lipids, like mono-palmitoyl, adopt a cubic phase system that is "infinite" in three dimensions. Once this cubic phase is established, membrane proteins can be introduced to the system and they will diffuse freely around the system. Hopefully the protein will make three dimensional contacts and pack into a crystal lattice. The researchers were able to produce crystals of bacterial rhodopsin (Landau 1996). The procedure is relatively straight forward. In a small tube, mix 10-20 microlitre of the protein solution and enough lipid to make a 60 - 70% (wt/wt) solution. I prepared a number of samples, varying the lipid percentage from 60, 70, 80 and 90. I also checked the protein concentration between the range of 3mg/ml to 6mg/ml, in addition to varying the amount water from 30-50% composition. All of this proved inconclusive. In fact, none of the samples even developed precipitate although a meniscus did form on some of the samples. I suspect that the infinite nature of the cubic phases may increase the solubility of the gramicidin, which may explain the lack of precipitate formation.

CHAPTER 20

CRYSTALS

Although the alteration of salts, metals, and pH had not proved successful, the method of seeding was showing some promising results. Experiments set up to duplicate the original experiment refused to yield crystals spontaneously. It has always seemed odd to me, that those crystals developed after 5 weeks. A solution of methanol and water can easily equilibrate in a day or two, yet crystals grew long after the equilibration period. So the question arises: what could have caused the shift in equilibrium to favour crystallisation? In truth, there are a number of factors which might have contributed. Despite our idealised view of a vapour diffusion experiment, we must remember that the system is constantly re-equilibrating to minor environmental factors like temperature change as a result of people entering and leaving the crystallisation room. These are the type of factors that cannot be controlled but must be accounted for whenever possible. So although I cannot accurately speculate on why wells of exactly the same conditions did not grow crystals, I can attempt to change it. This was accomplished through seeding techniques. In order to determine the optimal level of precipitant[water] for seeding, wells composed of between 30 and 50% water were streak seeded from the original conditions. Since these seeds had grown at 45% water, no crystals developed in wells lower than 45% water. Presumably the higher levels of methanol would dissolve the crystals. For this reason most of the initial streak seeding experiments were concentrated within the range of 44-50% water. When

these wells were streak seeded, they produced similar microcrystals to the original conditions but slightly smaller[*see INSERT figure 19*]. The problem being that it almost always resulted in a shower of microcrystals, despite the percentage of water. I had also tried dilution methods, but the crystals still developed to very small sizes. This signalled that a change in precipitant was not sufficient to change the size of these crystal. The only remaining components of the system to manipulate were the detergent and the protein.

It must be considered that these two component are quite interdependent. For example, if we increase the protein concentration and keep the detergent level constant, than we have really changed two parameters at once. In order to avoid this problem, I tracked both the raw protein concentration in mg/ml and the molar ratio of protein to detergent. The protein concentration was varied form .5 mg/ml to 12mg/ml, while the protein-detergent molar ratio varied from 1:11 to 1:88. Although some of these conditions did produce crystals without the addition of external nuclei, a large majority required seeding. A summary of the crystals, size, shape, and conditions follows:

Figure 20: Table of Conditions And Resulting Crystals

Gramicidin (mg/ml)	Gramicidin/ LDAO	% Water	Size µm	Description of crystals
2.2	1/44	50	15x75	cluster of needles
2.2	1/44	35	--	microcrystals
2.2	1/44	50	--	microcrystals
2.2	1/44	50	10x45	daggers
2.9	1/44	50	--	microcrystals
2.9	1/44	45	15x20	Wider needles than above
2.9	1/44	45	25x75	Rectangular needles*
2.9	1/44	45	--	microcrystals
2.9	1/44	40	25x100	rectangular needles
2.9	1/44	45	30x150	Rectangular needles(melted)
2.9	1/44	50	20x50	twinned rectangular needles(melted)
2.9	1/44	48	20x50	clusters*
2.9	1/44	48	20x100	faces not smooth*
2.9	1/44	49	15x75	Clusters a few single*
3.2	1/66	55	20x75	Twinned clusters
3.2	1/88	45	--	Twinned clusters
3.2	1/88	50	--	Rectangular and precipitate
3.2	1/88	40	5-35 x 115	shovels
3.2	1/88	50	25x75	Twinned, but some single
3.2	1/88	45	--	microcrystals
3.2	1/88	50	25x50	clusters on fibre

* indicates a result of seeding

Despite the large amount of information in this table, some of the important points to note are the trends in conditions which favour crystal growth. For example, protein concentration seems to be predominantly around 3mg/ml with a few exceptions. With respect to the protein/detergent ratio, no crystals form below the 1/44 cut off point. Despite the fact that lower amounts of detergent[1/11] are still above the critical micelle concentration (Ducruix and Geige 1992). Some data are not included in this table, like all the conditions that

produced a shower of microcrystals after seeding. Even with very low dilutions of seed crystals, the number of nucleation sites is very high. Also not included in the chart are other experiments that did not produce crystals. For example, β-octyglucoside was tested as an alternative detergent to LDAO, but only resulted in very chunky precipitate. Also, I tried cross seeding the seeds into pre-equilibrated well containing various salts, but to no avail.

This brings me to the highlighted condition of the chart. First it must be noted that the largest (30x150), most regular crystals met with an untimely demise. In an effort to prove they were protein, I stained the wells with coomassie blue. Unfortunately, this shifted the equilibrium inducing phase separation and the crystals on the fibre began to melt. The only image I have of these is after the stain had been added. Luckily, seeds form this well had been grown in a new well and eventually led to the 25x75 rectangular crystals [highlighted in blue]. Diffraction of these crystals was attempted in house, but problems with the detectors resulted in no data. The next crystals [highlighted in green] developed in the well next to the one that melted, but much later. This was rather a nice surprise to find while lamenting over their melted neighbours. Their size was a major problem, not only did they require very small cryoloops, but it was difficult to lasso a single crystal. The final set of crystals [highlighted in red], are only of interest for their strange geometry[*see INSERT, figure 21*]. They were shaped like tiny shovels complete, with a long handle attached to a cone. The cone was not a result of feathering, instead it was solid and regular in size from crystal to crystal.

Although the internal angles make these crystals useless for the purpose of diffraction, there structure and regularity was quite fascinating.

Looking back at the crystallisation conditions, it seems that although the seeding technique originally seemed quite promising, it did not produce the largest crystals. In fact, the largest crystals, which eventually melted, formed from internal nucleation not seeding. Despite this fact, one single crystal amongst all the other twinned rectangular ones was found in the seeded well highlighted in blue above. This crystal was taken to the Synchrotron Radiation Source at Daresbury National Laboratory for testing. It measured 25 by 75 microns and was very thin. Although this was below the normal limits for size, it was the best single crystal I had. Much to my dismay, this crystal did not make the journey well. When we arrived at Daresbury, all the contents of the drop had shifted. I do not know if it cracked while searching for it in the well, or sometime during the journey. As a result, the crystal which was placed in the beam was twinned or cracked and this was evident in the first frame collected. Since the twinning or fracture was severe, we decided that it would not be worthwhile to collect any more data. After this occurrence there was no more available synchrotron time in the remaining time that I had and none of the crystals were large enough for in house diffraction.

CHAPTER 21

THE DIMER

The last issue I would like to address in this discussion is the behaviour of the covalently linked gramicidin. After finding the 45% water condition for gramicidin A, I tried similar conditions for the dimer. It consistently precipitated out at 36% water to methanol. Since the shape of the dimer is longer than the monomer, I tried varied levels of LDAO and β-octyglucoside to determine if a different concentration of detergent would produce a more appropriate micelle. Alas, these detergents did not seem to change the nature of the precipitate. In addition, I used the additive screen to test a wide range of compounds in low concentrations, but they only changed the solubility slightly. At this point I had very little dimer sample left and had to cease work on this compound. Due to the limited amount of dimer, I was not able to examine a full range of pH, salts, temperatures, detergents or peptide concentration. Unfortunately, the dimer seemed to have a much more consistent behaviour than the monomer. For example, the monomer of gramicidin was soluble in a range of 40-50 % water/methanol solutions, whereas the dimer precipitates at precisely 36% water/methanol. This might have proved very useful in crystallisations, but there was not enough of the dimer to focus on this aspect of its behaviour

CLOSING REMARKS

Although the above research did not provide a three dimensional image of the channel conformation of gramicidin A, it has elucidated some of the finer points of the solubility, especially concerning factors like solvent, pH, salts, precipitants, detergents, and protein concentration. It has also shown that the covalently linked gramicidin dimer has a distinctly different solubility footprint. A thorough understanding of the solubility of a compound can greatly facilitate the crystallisation. It is my hope that this study of the crystallisation of gramicidin A will provide insight into the solubility behaviour and eventually contribute to crystallogenesis of the channel conformation of gramicidin.

BIBLIOGRAPHY

Arispe, N., Rojas, E., and Pollard, H. B. (1993) Alzheimer's disease amyloid beta protein forms calcium channels in bilayer membranes: Blockade by trimethamine and aluminum. *Proceedings of the National Academy of Sciences* 90: 567-571.

Arseniev, A. S., Lomize, A. L., Barsukov, I. L., Bystrov, V. F. (1990) Gramicidin A transmembrane channel: Three dimensional structural rearrangement based on NMR spectroscopy and energy refinement. *Biological Membranes* 3: 1723-78.

Barret, R.. E. W., Weiss, L. B., Navetta, F. I., Keoppe, R. E., Anderson, O. S. (1986) Single-Channel studies on linear gramicidin with altered amino acid side chains. *Biophysical Journal* 49: 673-86.

Berne, B. J. and Pecora, R.. (1976) *Dynamic Light Scattering.* John Wiley & Sons, Inc. New York.

Blundell, T. L. and Johnson, L. N. (1976) *Protein Crystallography.* Academic Press.

Bragg, W. L. and Bragg, W. H. (1924*) X-rays and Crystal Structure, Fourth Edition.* G. Bell and Sons.

Busath, D. (1993) The use of physical methods in determining gramicidin channel structure and function. *Annual Reviews in Physiology* 55: 473-501.

Cannon, S. J. (1996) Ion channel defects and aberrant excitability in myotonia and periodic paralysis. *Trends in Neuroscience* 19: 3-10.

Chiu, S. W., Jacobson, E., Subramaniam S., McCammon, J. (1991) Time correlation analysis of simulated water motion in flexible and rigid gramicidin channels *Biophysical Journal* 60: 273-285.

Cystic Fibrosis Foundation. (1997) http://208.196.156.141/

Diller, T. C., Shaw, A., Stura E. A., Vacquer V. D. and Stout, C. D.(1994) Acid pH Crystallisation of the Basic Protein Lysin from the Spermatozoa of Red Abalone. *Acta Crystallographica D*50: 620-626.

Dimer site. Http://voth.chem.utah.edu/~karger/channel.gif

Ducruix , A. and Geige, R. (1992) *Crystallization of Nucleic Acids and Proteins: A Practical Approach.* IRL Press at Oxford University Press, New York.

Dugandzija-Novakovic, S., Koszowsk, A. G., Levinson, S. R. and Shrager, P. (1995) Clustering of Na channels and Node of Ranvier Formation in Remyelinating Axons, *Journal of Neuroscience* **15(1)**: 492-503.

Eisenman, G. and Horn, R. (1983) Ionic Selectivity Revisited: The Role of Kinetic and Equilibrium Processes In Ion Permeation Through Channels. *Journal of Membrane Biology* **76**: 197-225.

Emery, A. E. H.. http://www.sonnet.co.uk/rupert/myotonia.html

Forsythe, E., Ewing, F. and Pusey, M. (1994) Studies on the Tetragonal Lysozyme Crystal Growth. *Acta Crystallographica D*50: 614-619.

George, A. and Wilson, W. W. (1994) Predicting Protein Crystallization from a Dilute Solution Property. *Acta Crytallographica D*50: 361-365.

Glusker, J. P. and Trueblood, K. N. (1985*) Crystal Structure Analysis: A Primer, Second Edition*. Oxford University Press: New York.

Harris L. J., Slalestky, E and McPherson, A. (1995) Crystallisation of intact monoclonal antibodies. *Proteins: Structure, Functions, and Genetics* **23**: 285-289.

Hotchkiss, R. D., and Dubos, R. J. (1940) Fractionation of the bactericidal agent from cultures of soil bacillus. *Journal of Biol. Chemistry* **132**: 791-792.

Holtzclaw, H. F., Robinson W. R., Odom, J. D. (1991) *General Chemistry*. D. C. Heath and Company: Lexington Massachusetts.

Hayward, L. J., Brown, R .H., and Cannon, S. C. (1997) Slow Inactivation Differs Among Mutant Na Channels Associated with Myotonia and Periodic Paralysis *Biophysical Journal* **72**: 1204-1219.

Image site. Http:/www.virtualchemistry.com/artgallery/gram.gif

Jolles, P. and Berthou, J. (1972) *FEBS Letters* **23**: 21.

Konnert, J. H., D'Antonio, P. and Ward, K. B. (1994) Observation of Growth Steps, Spiral Dislocations and the Molecular Packing on the Surface of Lysozyme Crystals with Atomic Force Microscope. *Acta Crystallographica D*50: 603-613.

Landau, E. M. and Rosenbusch, J. P. (1996) Lipidic Cubic Phases: A novel concept for the crystallisation of membrane proteins. *Proc. Natl. Acad. Sci. USA*. **93**: 1432-35.

Langs, D. A., Smith, G. D., Couseille, C., Precigoux, G. and Hospital, M. (1991) Monoclinic, uncomplexed double stranded antiparallel left- handed b 5.6-helix structure of gramicidin A: alternate patterns of helical association and deformation. *Proc. Natl. Acad. Sci. USA* **88**: 5345-49.

Langs, D. A. (1988)Three dimensional structure at .86 Å of the uncomplexed form of the transmembrane ion channel peptide gramicidin A. *Science* **241**: 188-91.

Lichtenberg D., Robson, R. J. and Dennis, E. A. (1983) Solubilization of Phospholipids by Detergents: Structural and Kinetics Aspects. *Biochimica and Biophysica Acta* **737**: 285-304.

Malkin, A. and McPherson, A. (1994) Light-scattering Investigation of Nucleation Processes and Kinetic of Crystallization in Macromolecular Structure. *Acta Crystallographica D*50: 385-395.

Masotti, L., Spinsi, A., and Urry, D. W. (1980) Conformational studies on the gramicidin A transmembrane channel in lipid micelles and liposomes. *Cell. Biophysical* **2**: 241-251.

McPherson, A. (1982) *Preparation and Analysis of Protein Crystals.* Wiley Interscience.

Michel, H. (1991) *Crystallization of Membrane Proteins* CRC Press, Inc.: Florida.

Neher, E and Eible, H. (1977) The influence of phospholipid polar groups on gramicidin channels. *Biochimica at Biophysica Acta* **464**: 37-44.

Neugebauer, J. M. (1990) Protein Purification: Membrane Proteins- detergent solubilization. *Methods in Enzymology.* **182**: 239-253.

Northrop, J. H., Kunitz, M. and Herriot, R. M. (1948) *Crystalline Enzymes.* Columbia University Press: New York.

O'Connell, A. M., Koeppe, R. E. Anderson, O. S. (1990) Kinetic of gramicidin channel formation in lipid bilayers: transmembrane monomer association. *Science* **250**: 1256-59.

Parker, J. (1997) http://dept.physics.upenn.edu/courses/phys501/khlin/node1.html.

Pecora, R., (1964) *Journal of Physical Chemistry* 40: 1604.

PI site(1997)- http://expasy.hcuge.ch/ch2d/pi_tool.html 82-187.

Ralston, G. (1993) *Introduction to Analytical Ultracentrifugation.* Beckman: Fullerton, California

Sarges, R. and Witkop, R. (1965) The structure of valine and isoleuine gramicidin A. *Journal of the American Chemical Society* **87**: 2011-20.

Scheffzek, K., Kabsch, W., Schilichting, I., Pai, E. F., Laytwein, A., Frech, M., Wittinghofer, A. and Goody, R. S. (1994) Crystallization and Preliminary X-ray Structure Analysis of Thermally Unstable $p21^{H\text{-}ras}$ Guanosine Complexes. *Acta Crystallographica D* **50**: 521-525.

Scopes, R. K. (1994) *Protein Purification: Principles and Practice.* Springer-Verlag, New York.

Sivaraman J., Coulombe, R., Magny, M., Mason, P., Mort, J. and Cygler, M. (1996) Crystallization of rat procathepsin. *Acta Crystallographica D***52**: 874-875.

Smart, O. S., Goodfellow, J. M. and Wallace, B. A. (1993) The Pore Dimensions of gramicidin A. *Biophysical Journal* **65**: 2455-2460.

Sousa, R. and Lafer, E. F. (1990) *Methods: A Companion to Methods in Enzymology vol 1.* IRL Press Oxford University Press 50-56.

Spinelli S., Ploux, O., Marquet, A. Anguille, C., Jelsch, C., Cabillau, C. and Martinez, C. (1996) Crystallization and Preliminary X-ray study of 8-amino-7-oxopelargonate synthase form *Bacillus sphaericus. Acta Crystallographica D***52**: 866-868.

Stankovic, C. J., Delfino, J. M. and Schreiber, S. (1990) Purification of Gramicidin A. *Analytical Biochemistry* **184**: 100-103.

Stankovic, C. J., Heinemann, S. H. and Schreiber, S. (1990) Immobilizing the gate of a tartaric acid-Gramicidin A Hybrid Channel Molecules by Rational Design. *Journal of the American Chemical Society* **112**: 3702-3704.

Steitz, T. A., Stenkamp, R. E., Geisler, N., Weber, K and Finch, J. (1981) X-ray and E. M. studies of crystal of core lac repressor protein. *Biomolecular Structure, Confirmation, Function and Evolution,vol 2* Pergamon Press: New York.

Stryer, L. (1975) *Biochemistry Third edition.* W.H. Freeman and Company, New York.

Stura, E. A., Kang, A. S., Stefanko, R. S., Calvo, J. C., Kalsow, D. C. and Satterthwait, A. C. (1994) Crystallization, Sequence and Preliminary Crystallographic Data for the Transmission-Blocking Anti-Malarial FAB 4B7 with Cyclic Peptides from the Pfs25 Protein of *P. falciparum. Acta Crystallographica D***50**: 535-542.

Stura, E. A., Satterthwait, A. C., Calvo, J. C., Kalsow, D. C. and Wilson, I. A. (1994) Reverse Screening *Acta Crystallographica D***50**: 448-456.

Thomas B. R., Vekilov, P. G. and Rosenberger, F. (1994) Heterogeneity determinations of commercial Hen Egg-White Lysozyme *Acta Crystallographica D*50: 776-784.

Urry, D. W. (1971) The gramicidin A transmembrane channel: A proposed π(L,D)helix. *Proc. Natl. Acad. Sci. USA* **68**: 672-676.

Veatch, W. R., Fossel, E. T. and Blout, E. R. (1974) The confirmation of gramicidin A. *Biochemistry* **13**: 5249-56.

Veesler, S., Marco, S., Lafont, S., Astier, J. P., and Boistelle, R. (1994) Influence of Polydispersity on Protein Crystallization: A Quasi Elastic Light Scattering Study Applied to α-Amylase. *Acta Crystallographica D*50: 355-360.

Wallace, B. A. and Ravikumar., K (1988) The gramicidin pore: crystal structure of a cesium complex. *Science* **241**: 182-191.

Wallace, B. A. and Janes, R (1991) Co-crystals of Gramicidin A and Phospholipid. A System for studying the structure of a transmembrane channel. *Journal of Molecular Biology* **217**: 625-627.