Instruction for the crystal growth practical

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1 Introduction

1.1 Motivation

Proteins are a very important class of substances in biochemistry. Both in the human organism and in other living beings they fulfil many functions. Notably, enzymes have to be mentioned. They are catalysts for chemical reactions and play a decisive role in digestion. The lysozyme used in the experiments described in the following is an enzyme which is part of the human immune system. Because of its antibacterial effect it is for example also contained in hen eggs. This effect bases on the catalysis of the splitting of a bond in peptidoglycan, which is a part of bacterial cell walls [1]. Another prominent example for physiologically relevant proteins is hemoglobin which is responsible for the

oxygen transport in blood. But also in industry, synthetically produced enzymes are applied, for example in cleaning agents in order to decompose contaminations [2] or as an additive in food [3]. The structure of proteins is connected closely to their function. But even in our days, the exact determination of a protein structure is still a challenge. For x-ray structure analysis, which is mostly used for this purpose, it is necessary to crystallize the protein. However, many proteins do not crystallize or only crystallize under very special conditions. Therefore, many conditions have to be tested in order to find one where crystallization occurs. Protein crystallization thus has a big relevance for experimental research.

1.2 Classical nucleation theory

The classical nucleation theory (CNT) was developed in the 1920's and provides a comparatively simple description of the crystallization process. Considering only the homogeneous nucleation, surfaces (like the walls of the container) and impurities which could influence the nucleation process are neglected. For the formation of a nucleus, it is necessary that during the collisions of the growth units swimming in the supersaturated solution, particles stick to each other and small clusters are formed [4]. We are talking about „growth units“ here because this can be either single molecules or oligomers.

The growth of the cluster is described by its free energy G with respect to the solution of particles. The energy, in turn, is determined by the (favourable) formation of bonds inside the cluster — a term proportional to its volume (G_V which is a negative term, also known as the bulk enthalpy of the cluster) — and by (unfavourable) unsaturated bonds on the cluster surface, thus proportional to its surface area (G_S).

The free energy of the cluster can thus be described as the sum of these two contributions:

$$G = G_V + G_S \tag{1}$$

The surface contribution term can be rewritten as

$$G_S = A \cdot \gamma \tag{2}$$

A is the surface of the cluster, γ the specific surface tension.

The volume energy, that means the energy that is gained when a particle from the solution accumulates to the cluster, can be expressed as the difference between the chemical potentials μ_α and μ_β of the solid and the solution multiplied with the number of growth units of which the cluster consists:

$$G_V = n \cdot (\mu_\alpha - \mu_\beta) = n \cdot \triangle\mu \tag{3}$$

The chemical potential can be expressed by the activity of the particular substance in the particular phase:

$$\mu_i = kT \cdot ln\, a_i \tag{4}$$

Hence, the volume energy can be written as:

$$G_V = nkT \cdot ln\left(\frac{a_\alpha}{a_\beta}\right) \equiv -nkT \cdot ln\, S \tag{5}$$

Here, the supersaturation ratio S is introduced, which is defined as the ratio between the real concentration and the concentration of a saturated solution. The activities can be approximately replaced by the concentrations. Altogether, G can thus be written as:

$$G = -nkT \cdot ln\, S + A \cdot \gamma \tag{6}$$

Now, one can write the free enthalpy either as a function of the number of particles n or as a function of the cluster radius r:

$$G_n = -nkT \cdot ln\, S + \sqrt[3]{36\pi\nu^2 n^2} \cdot \gamma \tag{7}$$

$$G_r = -\frac{4\pi r^3}{3\nu} \cdot kT \cdot ln\, S + 4\pi r^2 \cdot \gamma \tag{8}$$

ν represents the molecular volume of particles within the clusters.

In the following, we will focus on the second expression. By determining its maximum, we obtain the so-called critical radius of a cluster:

$$r^* = \frac{2\gamma}{kT\nu \cdot ln\, S} \tag{9}$$

For small clusters below r^*, the few interparticle bonds formed cannot compensate for the large amount of unsaturated surface bonds. For these clusters, the probability of dissolution is therefore quite high. Their (relatively improbable) growth results only from stochastic density fluctuations of the solution. However, once a cluster with $r = r^*$ is formed, the favourable volume term compensates the unfavourable surface term at the maximum of the cluster free energy (see. Fig. 1).

After this point, the volume term dominates the cluster free energy. Thermodynamically speaking, the free energy difference between a cluster with radius $r^* + dr$ and between one with radius r^* is negative ($\Delta G < 0$). Now, a reduction of the cluster size would be unfavourable because for the cluster to shrink, the energy barrier indicated by ΔG_i^{CNT} in Fig. 1 would have to be overcome. Cluster growth is thus favoured over cluster dissolution.

Once G crosses the x-axis and becomes negative, the formation of a crystal becomes energetically more favourable than keeping the clusters in solution. Crystal growth is thermodynamically uninhibited after this point.

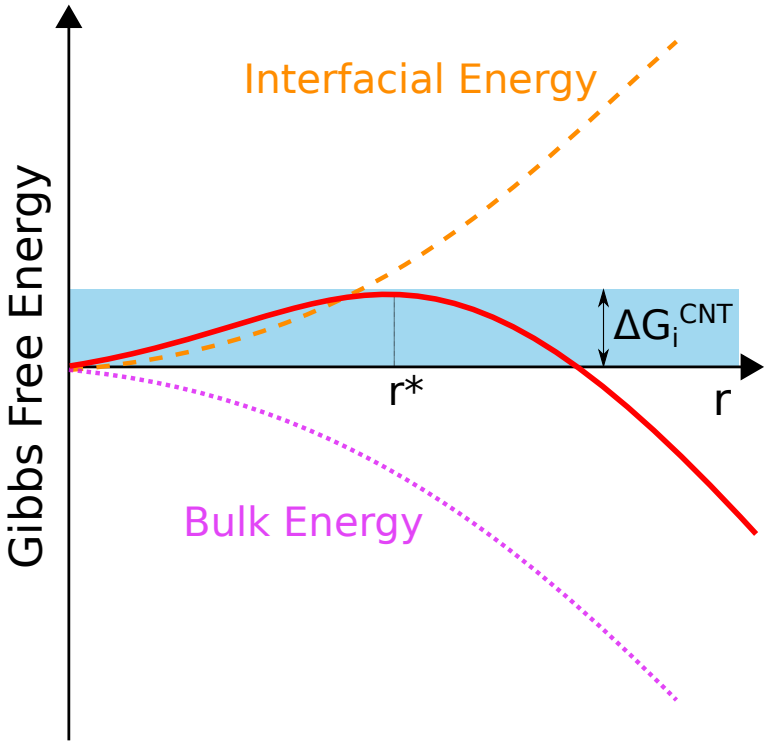

Figure 1: Free enthalpy of a cluster plotted against its radius. Cluster growth is no longer inhibited after the critical radius r^*. For $G < 0$, crystal formation is thermodynamically more favourable than maintaining the supersaturated cluster solution. Image courtesy of Andrea Sauter.

For the description of real crystallization processes, the classical nucleation theory makes some assumptions [5]:

- The clusters are spherical and clearly separated from the surroundings, the density within the cluster is constant and does not depend on the size. Hence, the molecular structure is also independent of the size.
- The specific surface tension γ (see equation 2) is independent of the size and the shape of the cluster. It is assumed that the surface tenstion of the cluster equates to the surface tension of a plane surface with the same surface area.
- A cluster can only grow through the addition of single growth units. There are no collisions between clusters during which clusters can fuse or be destroyed.
- The clusters are assumed as incompressible, the growth units in the surroundings as an ideal gas. This „gas“ has a constant pressure which does not change during the crystallization process.
- The nucleation rate is assumed as time-independent. The stationary state is reached immediately after a stationary state is existent.

1.3 Two-step nucleation theory

The classical nucleation theory is based on many simplifications and is not always a good model for the real nucleation process. For example, with some proteins — also with the lysozyme used in this practical — one can observe the so called „liquid-liquid phase separation“ (LLPS). During this process, before the crystallization, droplets with higher protein concentration than the surrounding solvent are formed. The crystals develop from these droplets. The crystallization thus takes places in two steps. This can be demonstrated in a diagram where the structure is plotted against the concentration (Fig. 2). In this context, concentration is also used as a synonym for the particle density within the cluster. During the classical nucleation process, the changes in concentration and structure happen simultaneously, whereas during the two-step process at first the concentration increases locally — the dense droplets are formed — and in the second step, the crystalline structure develops.

LLPS can be induced by additives such as depleting agents (e.g. polyethylene glycol, PEG) or salts. Another parameter that can influence LLPS is temperature. LLPS can occur either below or above a certain temperature threshold (the so-called critical temperature). The temperature at which the LLPS starts can be referred to as the cloud point temperature T_{cloud} because at this point the solution becomes very turbid and „cloudy“.

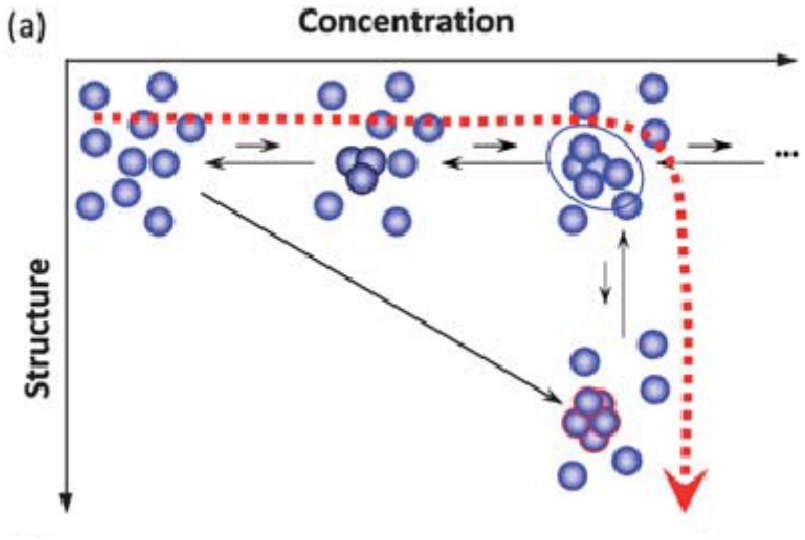

Figure 2: Classical and two-step crystallization in a structure-concentration diagram. [8]

The dense liquid is a phase which is metastable with respect to crystal phase. The dense liquid phase can be either metastable or stable with respect to the dilute phase, as you can see in figure 3. In most cases, the dense liquid is metastable both with reference to the dilute liquid and with reference to the crystal phase [8]. The rate-determining

step is the formation of crystals from the dense liquid. This is clear because one can observe the LLPS immediately after T_{cloud} is reached whereas the formation of crystals from the dense liquid droplets happens on a time scale of hours.

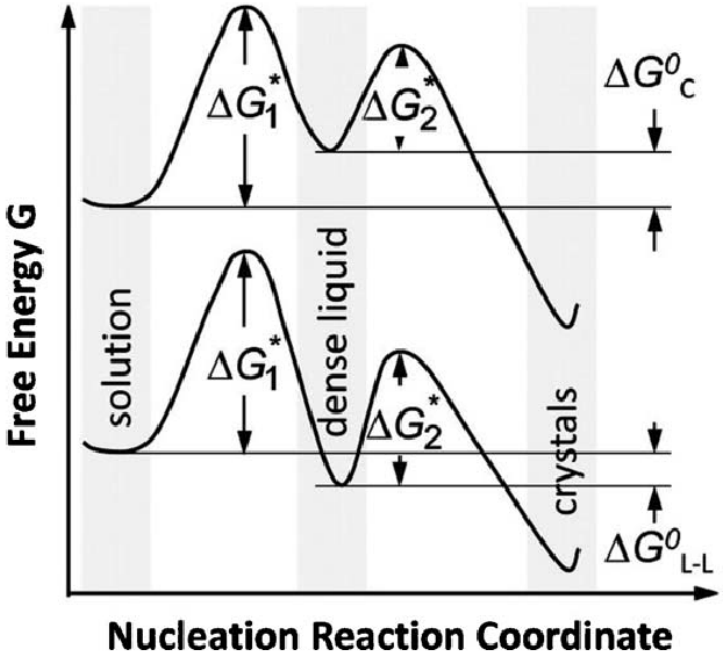

Figure 3: Free enthalpy during nucleation [9].

The Two-step nucleation process has also consequences for the crystal morphology. The so called „sea-urchin“ crystals (figure 4) are typical for the two-step pathway. Because of the high protein concentration, the crystallization within the dense liquid droplets happens quite fast. When the protein contained in the droplets has completely crystallized, crystallization proceeds outside, where the liquid is also a supersaturated solution. There is an advantage for those crystals which grow radially outward because the ingress of new protein is better in this case [10].

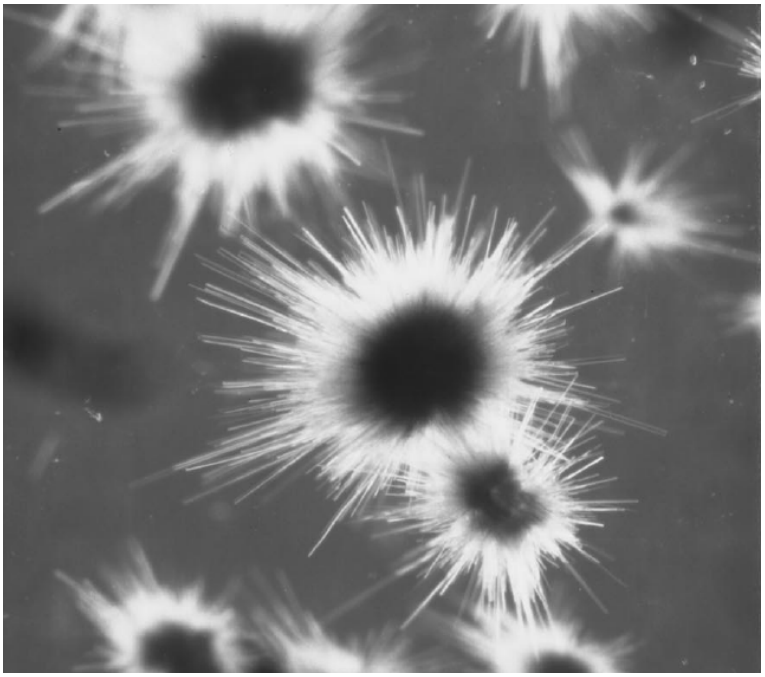

Figure 4: A „Sea urchin“ crystal [10].

1.4 DLVO theory

The DLVO theory was developed in the 1940's by the scientists Derjaguin, Landau, Verwey and Overbeek and describes the forces that influence the stability of colloidal particles - for example clusters that appear at the beginning protein crystallization. The potential that describes the interaction between two protein molecules consists of three contributions:

- The hard sphere interaction. The protein molecules can be considered as hard spheres in a rough approximation. When these „spheres“ touch each other, there is a strong repulsive interaction. On the molecular level, this can be explained by the fact that the electron clouds of the two proteins would overlap at a very short distance. Without a touch between the spheres, there is no interaction.

- The attractive van der Waals interaction. This interaction is omnipresent in chemistry and is not only important for proteins but for all organic molecules. It is an attractive interaction between molecules that have an electric dipole moment. There are three possibilities: either both molecules have a permanent dipole moment and attract each other when these dipoles are aligned antiparallel or one molecule has a permanent dipole moment and the other one is polarizable. The third possibility is that none of the two molecules has a permanent dipole moment but when there is a temporary dipole moment by accident, the other molecule is polarized and there is an attraction. The potential of the van der Waals interaction

is short ranged:

$$V = -\frac{C}{r^6} \tag{10}$$

In a very large macromolecule like a protein, this simple equation is not valid. For globular proteins, the following formula is correct [6]:

$$V(r) = -\frac{A_H}{12}\left(\frac{\lambda^2}{r^2} + \frac{\lambda^2}{r^2-\lambda^2} - 2ln\left(\frac{r^2-\lambda^2}{r^2}\right)\right) \tag{11}$$

- The repulsive Coulomb interaction. On a protein surface, a so called electric double-layer is formed. It consists both of charges that are localized directly on the protein surface, for example charged functional groups or ions that are solidly adsorbed on the surface and of ions that are not firmly bound but solved in the surrounding solution. On these unbound ions, there are two influences: The coulomb force from the surface charge and the diffusion caused by the thermal movement. The fixed charges are partially screened by the surrounding ions. The resulting potential is [6]:

$$V(r) = \frac{Z^2e^2}{\epsilon\cdot(1+\kappa a)}\cdot e^{-\frac{\kappa\cdot(r-\lambda)}{r}} \tag{12}$$

Z is the overall protein charge, ϵ is the dielectric constant of the solution, a is the radius and $\lambda = 2a$ is the diameter of the protein macromolecule. κ is a constant that is called Debye screening length. It is given by [6]:

$$\kappa = \sqrt{\frac{1}{k_BT\epsilon_0\epsilon}\cdot\sum_{j=1}^{M} e^2z_jn_j^0} \tag{13}$$

The index j refers to the different ion sorts in the solution, z_j is the charge of an ion of the sort j and n_j^0 the concentration of ions of the sort j outside the double-layer.

The resulting potential is plotted schematically in figure 5. The hard sphere interaction is neglected because it only occurs when the particles collide. As the attractive van der Waals interaction has a shorter range than the repulsive Coulomb interaction, there is a potential mountain that has to be overcome when particles approximate each other.

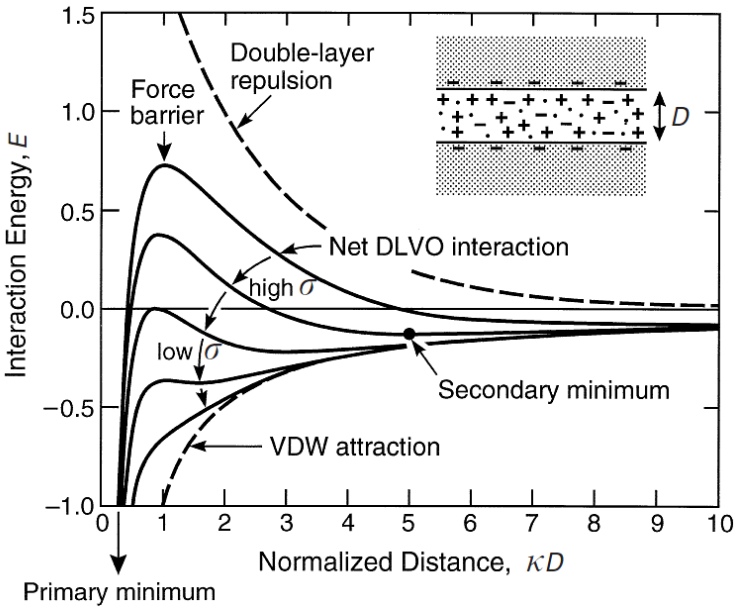

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Figure 5: Potential curves according to the DLVO theory. σ stands for the protein surface charge density [11].

2 Sample preparation

2.1 Preparation of the samples

The experiment starts with the preparation of the samples. The buffer solution, the protein stock solution and the sodium chloride stock solution (in this order) are pipetted into Eppendorf tubes and subsequently mixed using a Vortex mixer. The composition of the samples is listed in table 1. x is the concentration of the protein stock solution which varies from experiment to experiment. Therefore the volumes of the protein stocksolution as well as the ones of the buffer solution can only be given in dependence of x.

After mixing the samples are kept in a water bath (21 °C) for 30 min.

In the meantime the glass slides are prepared. Later, two or three droplets of each sample will be placed on the glass slide. Of each sample more than one droplet will be taken in order to prepare for the case that in one of the droplets no crystallization can be observed or that a cover slide breaks. For each sample two to three adhesive tape rings and cover slides are needed (ask your supervisor how many exactly). The cover slides are placed on a clean and dust-free laboratory wipe. For each droplet an adhesive tape ring is stuck onto a glass slide (three rings per glass slide). The numbers of the corresponding sample are written below each ring.

When 30 min have passed, 6 μl of the sample solution are placed in the middle of each tape ring. The cover slides are glued on top of the ring (you may use the vacuum gripper or tweezers).

Sample number	Salt concentration (% w/v)	Protein concentration ($\frac{mg}{ml}$)	Volume protein stock solution (µl)	Volume sodium chloride stock solution (µl)	Volume buffer solution (µl)
1	3,4	40	$\frac{8000}{x}$	58,3	$141,7 - \frac{8000}{x}$
2	3,9	40	$\frac{8000}{x}$	66,7	$133,3 - \frac{8000}{x}$
3	4,4	40	$\frac{8000}{x}$	75	$125 - \frac{8000}{x}$
4	4,9	25	$\frac{5000}{x}$	83.3	$116,7 - \frac{5000}{x}$
5	4,9	70	$\frac{14000}{x}$	83,3	$116,7 - \frac{14000}{x}$
6	5,4	70	$\frac{14000}{x}$	91,7	$108,3 - \frac{14000}{x}$
7	5,8	70	$\frac{14000}{x}$	100	$100 - \frac{14000}{x}$

Table 1: Composition of the samples

2.2 Microscopy

The most suitable magnification for the experiments described here is 20x.

2.2.1 Two-step-crystallization

To investigate the two-step-crystallization samples no. 5 to 7 have been prepared. Slightly different salt concentrations are needed because the time when the growth of the 'sea urchin'-crystals sets in cannot be predicted precisely. On the one hand it is possible that in sample no. 7 (highest salt concentration) the crystallization has already finished when the first to measurements have been done. On the other hand the crystallization in sample no. 5 may take more than five hours. Therefore the sample with the highest salt concentration which still shows LLPS should be chosen for further investigation. There should be nearly no crystals visible. One image is recorded before the following experiment (one-step crystallization); another image is taken after the one-step crystallization experiments are finished. Ideally, the same sample is chosen for both images.

2.2.2 One-step crystallization: growth process

If the temperature controlled microscope stage is used, it is set to 21 °C. Then the software for the camera, 'ZEN 2010' is started. One sample out of the sample series with numbers 1-4 is chosen after careful inspection together with the supervisor and placed under the microscope. As soon as a crystal is visible (which might already be the case when the observation is started), an automatic image series is started.

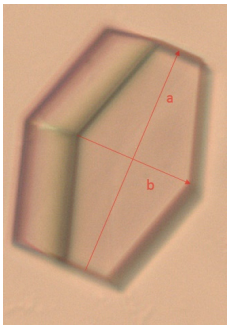

Figure 6: A tetragonal lysozyme crystal with its a and b axes labeled.

The recording time is set to 30 min. Each 5 min a picture is taken. When the image series for the first sample is finished, the same experiment is started for a different sample of the 1-4 series. **Important: the time points of sample preparation as well as starting and end points of the image series recordings need to be written down.**

2.2.3 One-step crystallization: crystal shape analysis

Three hours after preparation samples no. 1, 2 and 3 are compared. In each sample nicely shaped crystals are chosen and pictures are taken. For each sample all three droplets are examined. This means that in total nine pictures are taken.

3 Analysis

- **Two-step-crystallization** is slower than the classical crystallization. Choose pictures before and after the crystallization for your protocol to illustrate this phenomenon. Briefly describe and explain your observations.
- **One-step crystallization and growth process:** choose one well suited crystal from each image series and measure its axis lengths a and b (see Fig. 6). The software 'ZEN 2010' which you can use to do this is installed on the computer you use during the practical and is also freely available if you wish to refine your analysis at home. Plot a and b versus time for both crystals you choose and perform a linear regression. Calculate the growth rates $\frac{da}{dt}$ and $\frac{db}{dt}$ in $\frac{\mu m}{min}$. Calculate the crystal growth induction time t_0 from the linear regression.
- **Crystal aspect ratio analysis:** The pictures of samples no. 1 to 3 which were taken three hours after preparation are analysed as well. Measure the lengths a and b of several crystals from each sample and calculate their respective $\frac{a}{b}$ ratios. Calculate the average $\frac{a}{b}$ values and the standard deviation for each sample. Plot the average $\frac{a}{b}$ values and the error bars versus the salt concentration of the respective samples. Which tendency do you observe? Explain your observation.

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