Enhancing the Crystallisation of Insulin using Amino Acids as Soft-Templates to control Nucleation

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Amino acids have been widely used in protein formulations to increase the protein’s stability. In this study, amino acids have been introduced as soft-templates to control the nucleation of proteins. L-arginine, L-glycine or L-leucine with concentrations between 0.01 M and 0.1 M were used in a hanging-drop vapor diffusion set-up to investigate their role in the crystallisation of human insulin as a model protein at low supersaturation. All amino acids were in a dissolved state. Here we show that L-arginine and L-leucine clearly enhance the nucleation significantly. On the other hand, L-glycine does not enhance nucleation of human insulin at low supersaturation. We hypothesize that it is the intermolecular interaction between the protein’s residues and the amino acid’s residues that results in an enhancement in the formation of the initial protein nuclei. To prove that the enhanced nucleation is of a kinetic nature, the solubility of insulin in amino acid rich solution was also investigated. The solubility results show that amino acids increase insulin solubility. From that we derive that the enhancement in nucleation is not due to a change in the thermodynamic equilibrium between the crystalline and bulk-liquid phase. As this approach of using amino acids to enhance nucleation is based on a dissolved state in solution, we introduce here the concept of protein crystallisation by soft-templating.

1. Introduction

Since the first isolation and therapeutic use of the protein insulin for treating diabetes in the 1920s, the global polypeptide and protein drug market has grown significantly in size and economic value over the past decades, having a market value of $21.5 billion USD in 2016 and expecting a growth rate of 9.4% per annum for the coming 5 years until 2025 (1). Stemming from the rapid growth in demand, significant improvements in upstream processing, such as more efficient fermentation and cell culture technologies, lead to an increase in titre production which heavily relies on costly multi-step packed-bed chromatography (2) and accounts for a total of up to 70% of the total production costs (3).

Crystallisation as a purification technique is already widely adapted in the manufacturing of small molecules. Crystallisation is seen as an efficient technique since no mass transfer limitation exists, no costly equipment and consumables and less buffer solutions are required, while still achieving a high maximum product purity (4, 5). Not only is crystallisation a more economical and efficient technology compared to chromatography, but also offers the crystalline form pharmacokinetic advantages, such as better release control and higher bioavailability and lower impurity loading (6). However, a protein’s high number of residues and high structural flexibility paired with a lack in understanding the underlying physico-chemical mechanism of protein crystallisation makes proteins notoriously difficult to crystallise. Additionally, it is very difficult to control the crystallisation process and challenging to produce protein crystals with desired crystal qualities.

It is believed that hydrogen bonding, electrostatic and hydrophobic interaction, which all are governing the protein-protein interaction, are most crucial in protein crystallisation (5). The stochastic event of nucleation is the governing and rate-determining step in crystallisation due to simultaneous occurrence of favourable protein-protein interaction and a local increase of protein concentration to enable the formation of the critical nucleus. Hence, nucleation is an extremely complex process which can be affected by both thermodynamic and kinetic factors (7, 8). The complex interplay of attractive and repulsive short-range protein-protein interaction and the necessity of a threshold number of protein molecules to overcome the energy barrier and form the critical nucleus makes protein nucleation notoriously slow and uncontrollable. Hence, a major challenge in applying crystallisation is the lack of controllability of the nucleation mechanism.
The two most commonly used routes to achieve a controlled crystallisation is by controlling the level of supersaturation by either slowly decreasing the temperature (cooling crystallisation) or slowly increasing the content of precipitation-additives (salting-out) (9). However, those approaches are rather time-consuming and often have limited success. Hence, major efforts have been done to facilitate and control nucleation with external stimuli, such as utilising an electric field (10) or magnetic field (11), ultrasonic environment (12), microgravity (13), additional additives such as ionic liquids (14) or heterogeneous nucleants (15, 16).

Heterogeneous nucleants have been demonstrated to be relatively successful in facilitating nucleation by stabilising the protein in the surface vicinity and lowering the interfacial energy needed to form a critical nucleus. The interplay of these effects results in a significantly faster crystallisation and reduces the necessary bulk-supersaturation needed to form nuclei (15, 17, 18). Heterogeneous nucleants include mineral surfaces (19), membranes (20), polymers (21), DNA-origami (22), nano- and mesoporous particles (23, 24), porous surfaces (25, 26), and surfaces with selected functional groups (4, 17, 25, 27).

Selective nucleants that have an engineering surface topography and morphology, such as molecular imprinted silica surfaces or polymers, have the ability to selectively crystallise proteins due to favourable geometrical and physico-chemical interaction between the protein and the nucleant’s surface (4, 15, 18, 28, 29). We term those selective nucleants as hard templates, since the nucleant is rigid and does not dissolve in the crystallising solution.

In this study, we introduce the concept of soft templating, which describes the facilitation and control of nucleation and crystallisation with soft organic molecules. In comparison to hard templates, soft templates aim to match and enhance favourable protein-protein interaction and block unfavourable protein-protein interaction resulting in an enhancement of crystallisation by facilitating nucleation (Figure 1). Soft templates can be sub-grouped as either additives or heterogeneous nucleants. Additives are soft organic molecules that fully dissolve in solution whereas heterogeneous nucleants are soft organic molecules which provide a soft heterogeneous surface for nucleation (e.g. pores of a DNA origami tube). Thus far, dissolved organic polymers (30, 31) and 3-dimensional DNA foldamers (22) have been shown to facilitate nucleation.

Here, we would like to introduce natural amino acids as novel soft templates. Besides being the building block for proteins, amino acids are widely utilised as excipients in freeze-and spray-drying (32), as stabilising additives (32-34), as osmolytes (35) in liquid protein-based drug formulations or as stabiliser in crystalline formulations (36). In liquid protein formulations, hydrophobic and acidic hydrophilic amino acids have been used to thermally stabilise lysozyme (37) and pig heart mitochondrial malate dehydrogenase (32) respectively, while methionine and histidine increases the stability against oxidative stress to enhance the long-term storage capability (32, 38, 39). Furthermore, amino acids, especially arginine, have been used to prevent protein denaturation (32, 34, 40). Arginine (32, 41, 42), histidine (43), glutamic acid (42), isoleucine (42), glycine (44) and combination of arginine and glycine (44) have been found to stabilise and protect the stability of proteins, such as catalase, lysozyme and haemagglutinin proteins during freeze-drying.

We believe that amino acids as soft templates are remarkable in controlling nucleation due to their similarities in surface chemistry to the protein surface. Additionally, amino acids are small in size compared to the protein surface which enables targeted interaction with a specific surface patch of the protein. Hence, we hypothesise that amino acids have the potential to 1) specifically enhance protein-protein interaction leading to the formation of the initial nucleus or 2) block unfavourable interaction to enhance the likelihood of the formation of the initial nucleus.

To demonstrate the soft-templating approach, amino acids with specific residues and human insulin, which comprises of two unique chains, A and B, with a combined 51 amino acids was utilised. Insulin was utilised as a model protein since it comprises of amino acids with a wide variety of physico-chemical residues.

![Figure 1: Schematic showing amino acid enhancing the interaction between protein molecules (e.g. insulin) in the soft-templating concept resulting in an enhancement of nucleation and crystallisation.](image_url)
measured with a 4330 glass-bodied conductivity probe (Jenway, UK) conductivity meter. Zinc-sulphate and amino acids were separately dissolved in buffer solution. All solutions were filtrated with a 0.22 μm Millex-GS syringe filter units (Millipore) before further usage.

2.3. Solubility determination of insulin in aqueous amino acid solutions

The solubility of insulin in aqueous amino acid and zinc-sulphate solution was determined by adding a small amount of insulin crystals to the amino acid, zinc-sulphate and buffer solution. The total volume of each solubility samples was 5 ml. The solution temperature was kept constant by placing the vials in a water bath, set at a temperature of 24 °C and the temperature was monitored with a digital thermometer (Hanna Instruments, UK). The vials in solution were tightly sealed to prevent solvent evaporation and it was ensured that the sample in the vials were submerged in the water bath. The insulin crystals were obtained via batch crystallisation under the same conditions as the solubility of insulin was probed. Figure 2 shows the insulin crystals obtained from the batch crystallisation experiments that were added to the sample solutions. The insulin concentration in solution was determined by high performance liquid chromatography (HPLC) after 6 days. Full description of the chromatographic condition can be found in the Supporting Information.

![Image](image_url)

Figure 2: Rhombohedral insulin crystals obtained from batch crystallisation without amino acids. The conditions for the batch crystallisation matched the conditions in the sample for solubility determination and are the following: 0.48 M citric acid buffer, 0.7 mM zinc-sulphate and arginine, glycine or leucine concentration between 0.01 M and 0.1 M or without amino acids as shown here.

2.4. Crystallisation of human insulin

Hanging drop vapor diffusion experiments were carried out to investigate the impact of amino acids on human insulin crystallisation. Insulin crystallisation conditions were adapted from the literature and further optimized (e.g. type and pH of buffer and zinc containing salt) (45-48). The buffer solution, the zinc-sulphate solution and the amino acids solution were pre-mixed in an Eppendorf tube for every experiment to achieve homogeneity in all droplets. No crystallisation occurred in the pre-mix process. From the Eppendorf tube a 2 μL droplet was placed on a hydrophobic borosilicate cover glass and then carefully inverted and sealed onto the well filled with reservoir solution. The zinc-sulphate concentration in the reservoir was twice as high as in the droplets and the amino acid concentration in the reservoir was equal to the amino acid concentration in the drop. The 24-well plates were incubated at a constant temperature of 24 °C and monitored with an optical microscope (CX 41, Olympus, Japan) after 1, 2, 3, 4, 7, 10 and 14 days. A minimum of 72 drops from a minimum of 6 different plates were evaluated. Methylene blue dye was utilised to distinguish protein crystals from salt or amino acid crystals. It was demonstrated that pure amino acid crystals (arginine, glycine or leucine) are not stained by the methylene blue dye (Figure 3). Therefore, those amino acids were crystallised via cooling crystallisation in pure deionised water and in small batches.

![Image](image_url)

Figure 3: Crystals of amino acids in deionised water and insulin in citric acid buffer. Top left: glycine crystals; top right: arginine crystals; bottom left: leucine crystals and bottom right: human insulin crystals without amino acids. (Note: Scale bar is 100 μm for all images)

For simplification, only images of hanging drop vapor diffusion experiments in which crystallisation occurred are shown. The impact of amino acids on enhancing crystallisation was evaluated by measuring the occurrence of crystals (Equation 1) after 4 and 7 days.

\[ \text{Occ}_\% = \left( \frac{\text{Number of drops with crystal}}{\text{Total number of drops observed}} \right) \times 100 \]  

3. Results

3.1. Insulin crystallisation at low supersaturation

In order to probe the impact of amino acids as soft templates, an initial insulin concentration was chosen in which insulin was found to be difficult to crystallise due to the low supersaturation. As expected, the occurrence of insulin crystals decreases with decreasing insulin concentration at constant zinc-sulphate concentration. Figure 4 shows the insulin crystal occurrence at 3 distinct low initial insulin concentrations (c_i = 0) . The standard deviation for the occurrence shown in Figure 4 is ~ 12%. The solubility of insulin (c_o) at a zinc-sulphate concentration of 0.7 mM in a 0.48 M buffer is 0.011 mg/ml (see chapter 3.2). An initial insulin concentration of 0.2 mg/ml (supersaturation ratio \( S = c_i / c_o = 18.2 \)) was selected because lower concentrations would increase the impact of
external effects such as impurities, sample inhomogeneities or even inconstant drop size (49). On the other hand, a concentration higher than 0.2 mg/ml will increase the crystallisation of insulin within the control conditions and hence the impact of the amino acids on crystallisation will not be determinable.

Figure 4: Occurrence of insulin crystals after 4 days as a function of initial insulin concentration without amino acids. An insulin concentration of 0.2 mg/ml was chosen as control since the concentration is close to the metastable zone, but still within the nucleation region. The occurrence at this condition is 15.6%.

Glycine was chosen as the simplest amino acid with only one hydrogen as a residue to probe whether the amine or acidic functional groups interact with the amino acid residues of insulin. Leucine is often described as the most hydrophobic amino acid (50) while arginine is a basic amino acid commonly used in solid protein formulations as a stabiliser and also increases the solubility of proteins. Figure 5 presents the crystal occurrence of insulin crystals with the addition of different amino acids types and concentrations. The standard deviation for the crystal occurrence is between 0 to 20% for insulin concentration of 0.2 mg/ml (Figure 5) after 4 days. The standard deviation of the control case (~12%) is similar to the deviation for glycine (~15%), but slightly higher for leucine (~20%). For arginine as a soft template, the standard deviation is ~0% for 0.01, 0.03, 0.06 M and 17% for 0.1 M. The highest concentration used for each amino acid was 0.1 M, since the maximum concentration of leucine which can be dissolved in the utilised system was just slightly above 0.1 M. Figure 5 shows that arginine and leucine increase the crystal occurrence significantly from 15.6% (control) to 100% (arginine) and 73% (leucine), whereas the crystal occurrence in presence of glycine was around 10 - 33 % for all concentration probed. The exact values of the crystal occurrence in addition of arginine, glycine or leucine can be found in the Supporting Information. At an arginine concentration higher than 0.1 M, no crystallisation was observed (data shown in the Supporting Information). This agrees with studies from Shiraki et al., who showed that arginine concentration higher than 0.2 M prevented completely (100%) the aggregation of the basic protein lysozyme (34). The increase in crystallisation rate (increase of crystal occurrence within a set period of time, e.g. 4 or 7 days) when arginine is added can also be attributed to the pH increase triggered by the dissolved arginine molecules in the solution. Measuring the pH in the Eppendorf-tube before setting up the drops revealed that even 0.03 M of arginine increases the pH from 7 (control) to 9.5 due to the exceeding of the buffer capacity. It has been shown that a pH opposite of the protein’s pl, e.g. acidic pH for a protein with a basic pl (e.g. lysozyme) favours the protein’s crystallisation significantly, resulting in an improved crystallisation due to alteration of the solubility and supersaturation (49, 51). Therefore, a more basic pH might increase the nucleation rate for insulin (pl = 5.4).

Figure 5: Crystal occurrence of insulin as a dependence of arginine, glycine and leucine concentration after 4 days (top image) and 7 days (bottom image) of observation. 0.48 M citric buffer with 0.7 mM zinc-sulphate was utilised. Crystals in drops were stained with methylene blue dye to confirm that they were insulin crystals.

In the presence of leucine, the rate of nucleation increases (more drops with crystals are obtained in a set period of time,
e.g. 4 or 7 days) with increased leucine concentration. While the crystal occurrence with leucine at concentration of 0.01 – 0.03 M is not statistically different to the control, leucine at concentrations of 0.06 and 0.1 M has a significant effect on promoting nucleation (see SI for standard deviation). At leucine concentration of 0.1 M, which is just below the solubility of leucine in the initial crystallisation cocktail, leucine starts to crystallise out after ~56 days. Figure 6 shows sheet like leucine crystals in the presence of rhombohedral insulin crystals. This could also be an indication that leucine is not incorporated in the insulin crystals, but instead only promotes insulin nucleation. It also shows that amino acids and insulin can be individually and selectively crystallised out from the same solution. While arginine and leucine are able to enhance the crystallisation of insulin, adding glycine did not result in an enhanced insulin crystallisation. This results in the consideration that the amine and acidic group of amino acids are not significantly involved in the nucleation-promoting interaction between insulin molecules which are important in the nuclei formation. Additionally, glycine does not promote nucleation which contradicts previous findings in which glycine is described as “structure breaker” (52) and could have acted as a Hoffmeister salt leading to an enhancement in crystallisation due to salting-out.

Since the supersaturation is very low, the overall insulin crystals are significantly smaller than reported in literature (53). When no amino acids were added, insulin crystallised out by forming the typical rhombohedral crystal shape which has been reported in literature (53). Adding glycine or leucine does not alter the crystal shape even at concentration of up to 0.1 M. On the other hand, adding arginine results in insulin crystals of rhombic dodecahedral crystal shape. The rhombic dodecahedral crystal shape was obtained for all arginine concentration probed (see Figure 7 top row). With increasing arginine concentration, significantly larger insulin crystals were observed. Crystals obtained in the presence of arginine below a concentration of 0.1 M are significantly smaller in size than crystals obtained with glycine, leucine or even without amino acids, which indicates a rapid nucleation and a very limited growth.

3.2. Solubility of insulin in aqueous amino acid solutions

The impact of amino acid concentration and type on the solubility of human insulin was studied to investigate the mechanism of the soft-templated crystallisation. Figure 8 shows that the solubility of insulin increases if arginine, leucine or glycine is added with concentration between 0.001 and 0.1 M. While leucine or glycine increase the solubility of insulin of up to 3.2 folds, arginine increases the solubility significantly for concentration above 0.01 M to a maximum of 18.3 folds at arginine concentration of 0.06 M and 0.1 M. These findings agree with reported studies on the increasing effect of amino acids on peptide and protein solubility (48). The solubility also reveals that the effect of arginine and leucine is not due to a change in the thermodynamic equilibrium (solubility) but rather of kinetic nature. Hence, we hypothesize that both, arginine and leucine, promote favourable protein-protein interactions between insulin molecules which increases the likelihood of nucleation.
4. Discussion: Mechanism

4.1. General aspects

This study represents the first report of amino acids enhancing the nucleation of insulin at low supersaturation. Hanging drop vapor diffusion experiments were carried out and the initial concentration of amino acid within the drops was equal to the amino acid concentration in the reservoirs to exclude the impact of the amino acid on the partial pressure and the vapor diffusion rate.

While leucine and arginine have shown crystallisation-promoting effects, glycine did not improve the crystallisation. To evaluate the impact of amino acids on the crystallisation of insulin, the concentration of amino acid in the drop was equal to the concentration of the amino acid in the reservoir. Since the buffer salt concentration and amino acid concentrations are significantly higher than the zinc-sulphate concentration (0.7 mM), it can be assumed that the water diffusion ceased after a short period of time (< 4 days) since the occurrence does not change after 4 days. After 4 days, the drops behave as small (2 µl) hanging batch crystallisers. It has been shown that the air-liquid interface can impact nucleation significantly. Therefore it is important to mention that dissolved amino acids change the surface tension of the crystallisation-solution-air interface (50) and also alter the interfacial surface properties between the borosilicate cover glass and the crystallisation-solution. When arginine or leucine were added, the majority of crystals were observed in the centre of the drop, while for the control or glycine the majority of crystals were observed near the 3-phase boundary (air, cover-glass and crystallisation solution). Crystals which nucleate near the phase boundary do not sediment towards the centre of the drop but stay at the location where nucleation occurred (see Figure 9). Since the majority of nucleation with addition of arginine or leucine occurred in the centre of the drop (Figure 7 top and middle row), the enhancement of the nucleation rate is not due to the air-liquid interface. Although adding glycine changes the surface tension of the water-air interface (50), the majorities of crystals within the control and glycine experiment occurred at the air-liquid-glass interface, indicating that the change in surface tension due to amino acids does not increase the nucleation near this interface. Since the nucleation rate for the control or addition of glycine is lower than for arginine or leucine, the interface induced nucleation is less impactful compared to the amino acid induced nucleation in the bulk solution.

We also investigated the fundamental mechanism of amino acid induced protein crystallisation. According to the classical nucleation theory, the nucleation rate can be expressed with the following equation

\[
J = A \cdot \exp \left( -\frac{B}{ln^2 S} \right)
\]

(2)

Where parameter A describes the molecular attachment kinetics and parameter B is related to the nucleation barrier which has to be overcome. For ideal solutions, the supersaturation S can be expressed as \( S = \frac{C_i}{C_e} \), with \( C_i \) is the protein concentration at a certain point in time and \( C_e \) the equilibrium concentration (solubility). While B can be described by equation 3, A is a complex parameter and depends on the mass transfer from the bulk to the nuclei surface (e.g. volume-diffusion or interfacial-transfer) but is directly proportional to the diffusivity in a quiescent environment (54).

\[
B = \frac{16\pi \gamma \rho^3}{3(k_BT)^2}
\]

(3)

With \( \gamma \) being the specific surface free energy of the crystal cluster and bulk-solution interface and \( v_0 \) is the volume occupied by one molecule in the clusters.

Studying the solubility of insulin in amino acid rich solution confirmed previous studies which report that amino acids, especially arginine, increase protein solubility (32). If the supersaturation is not increased (i.e. solubility has increased by the addition of the amino acids), as observed in this study, the mechanism of amino acid enhanced nucleation must be due to either a reduction of the activation barrier of nucleation (parameter B) or an increase in the molecular attachment kinetic (parameter A).

4.2. Arginine

Although a pH of 7 is slightly higher than the pKa for citric acid (~6.3 pH), citrate buffer at a pH of 7 is widely utilised as buffer for insulin crystallisation (48, 55). However, adding arginine (>0.01 M) to the solution lead to a change in the pH of the crystallisation cocktail (from 7 to 9.5 pH) since the amount added exceeds the buffer capacity. That a change in buffer pH, as observed, has an effect on protein crystallisation has been widely studied in literature, as the pH is one of the most important parameters that governs crystallisation of proteins. By evaluating the relationship between solution pH and the protein’s isoelectric point (pl) for hundreds of proteins, it was found that for proteins with a basic (acidic) isoelectric point a buffer pH below (above) the proteins isoelectric point enhances the crystallisation (56, 57). Although this study finds that most of proteins successfully crystallise if the difference between solution pH and protein’s pl is between 1 to 3 pH units, it is also known that not every protein follows this trend. Lysozyme (pl = 10.7 pH) and human insulin (pl = 5.4 pH) are known to crystallise successfully at a buffer pH of 4 to 5 and 6 to 10 for lysozyme and human insulin respectively. Shifting the pH further away from the protein’s isoelectric point has been shown to increase nucleation rate and enhances the formation.
of well-built crystals instead of amorphous precipitates (51, 55, 58). Additionally, an increasing pH could also increase insulin diffusivity resulting from increased preferential interaction, as it has been shown for lysozyme (59, 60).

Increasing the pH towards a more basic pH also increases the net negative charge on the insulin surface resulting in higher electrostatic repulsion. Hence a reduction in self-association and nucleation would be expected. The guanidinium group of arginine (pKa = 12.5) has a positive charge at the pH at which crystallisation takes place (pH 9.5). At this pH, arginine binds to the negatively charged residues on the insulin surface, such as glutamine or histidine, and neutralises repulsive electrostatic interaction, leading to an enhanced insulin aggregation and crystallisation. Enhanced insulin aggregation due to the addition of arginine at pH around 7.5 have already been reported in other studies (61). At higher arginine concentration (>0.06 M), the crystal occurrence starts to decrease (e.g. occurrence at 0.1 M arginine is 81% after 4 days compared to 100% at 0.06 M arginine, see Figure 5 top). This could be due to 1) the increase in solubility as seen in Figure 8 and/or 2) the prevention of insulin aggregation and crystallisation at higher arginine concentration (>0.1 M) which is widely reported in literature (34, 60, 62).

We hypothesize that the combined effect of increased pH and addition of arginine reduces unfavourable (e.g. repulsive) interaction and enhances the attachment of insulin molecules during the formation of the critical nucleus in a controlled manner which depends on the arginine concentration. This can be expressed in equation 2 by an increase in parameter A and hence an increase in the nucleation rate. Besides promoting nucleation, the addition of arginine and the increase in pH also leads to a smaller mean crystal size, as it can be seen in Figure 7. The effect of a smaller crystal size by changing the pH is already reported in literature. However, our findings contradicts with recent research which proposed that a pH further away from the protein’s pI leads to the formation of bigger crystals as we obtain very small crystals for arginine concentration between 0.01 – 0.06 M at 9.5 pH (see Figure 7 top row) (58). The increase in crystal size obtained with an arginine concentration of 0.1 M could be due to the suppression of insulin aggregation, resulting in a low nucleation rate and hence large insulin crystals (see Figure 7).

Adding arginine leads to the formation of the rhombic dodecahedral crystal shape over the rhombohedral crystal shape, which we would have expected due to the addition of zinc. The formation of the rhombic dodecahedral shape has been shown to take place in a zinc free crystallisation environment in which a dimer is one growth unit (63). This change in crystal shape with the addition of arginine could originate from 1) arginine enhances the specific interaction that favours the formation of rhombic dodecahedra insulin crystals which small molecules are capable of (64). The specific interaction in this case refers to the interaction between the amino acid, acting as soft templates, with the insulin molecule. In order to crystallise within the rhombohedral crystal shape, three insulin dimers form a hexamer where the His10 of each dimer interacts with 2 Zn$^{2+}$ to form a hexamer. However, arginine at higher ionic strengths and concentration also interacts with the dimer at the His10 position which could lead to a replacement of the Zn$^{2+}$ ions with the guanidinium group of the arginine residues (65), leading to the zinc free rhombic dodecahedral crystal shape. Furthermore, it has been shown that the surface free energy of insulin crystals with the rhombic dodecahedral shape is lower than insulin crystals with a cubic shape (61), which could indicate a change in the interfacial energy between the critical nucleus and the bulk solution with the addition of arginine. Or 2) The increase in pH stemming from the protonated arginine molecules in solution promotes the formation of hexagonal insulin crystals. It has been shown that protein crystal shapes are strongly dependent on the pH of the solution and a change in the pH can promote or inhibit a certain crystal shape (e.g. altering the aspect ratio or crystal form) (58, 66).

4.3. Leucine

In comparison to arginine, leucine does not change the pH of the solution since leucine has a hydrophobic and not an electrically charged residue. According to Bull and Breese (50), increasing leucine concentration lowers the surface tension of the bulk solution. This indicates that the interfacial free energy at the crystal cluster and bulk-solution could be reduced and hence the energetic barrier of nucleation could be lowered (decrease of the activation barrier). Recent studies showed that amino acids can impact and alter the secondary structure of the native protein (67) which also indicates a change in the interaction intensity between the protein molecules and bulk liquid phase. Additionally, the promoting effect of hydrophobic additives has already been shown in literature (68-70). Since leucine is a hydrophobic amino acid and reduces the interactions between the water molecules, the addition of leucine might lead to an increase in the hydrophobic interactions. The hydrophobic interaction has been reported to be a dominant interaction within a dense liquid cluster prior to crystallisation (69). On the other hand, leucine could suppress unfavourable hexamer contacts that do not lead to the formation of ordered hexamer arrangements. Leucine acts by binding to hydrophobic residues not buried in the interior of the insulin hexamer. Unfavourable contacts could be the interaction of a hydrophobic surface patch and a hydrophilic one which does not lead to crystallisation (70). This could be mathematically expressed with an increase in the kinetic constant (parameter A in equation 2) resulting in an increased nucleation rate.

4.4. Glycine

Although glycine, and every other amino acid, is present in its zwitter-ion state in aqueous solution at neutral pH, and hence provides two opposite electrically charged groups, it was found that it does not promote crystallisation. Although the crystal occurrence of insulin in addition of glycine is higher than for the control, the difference is too small to derive an enhancing effect of glycine. Compared to arginine and similar to leucine, glycine does not change the pH nor the ionic strength of the solution.
Hence, it can be hypothesized that the amine and acidic group of amino acids play a minor role in facilitating crystallisation.

5. Conclusion

We have shown that amino acids have the propensity to control protein nucleation even at low supersaturation. Both arginine and leucine were found to enhance the nucleation, whilst glycine did not alter the nucleation of insulin. This increase in nucleation is not due to an increase in supersaturation as the addition of arginine resulted in an increase in the solubility of insulin, while leucine and glycine did not alter the solubility. The increase in nucleation is attributed to the promotion of attractive interaction and the suppression of unfavourable insulin hexamer contacts. Here, we demonstrated that the type and concentration of amino acid plays a significant role in the promoting effect.

Conflicts of interest

There are no conflicts to declare.

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