Chapter 3

Growth inhibition of protein crystals: A study of lysozyme polymorphs

Crystal morphology is determined by the relative growth rates of the different faces involved. Opposite faces (hkl) and $(\bar{h}\bar{k}\bar{l})$ can show different rates if the crystal structure does not have inversion symmetry. Protein crystals, being built of asymmetric molecules do not have identical opposite faces, except for those pairs linked by rotational symmetry. Here, we present an in-situ microscopy study on the polar growth of various polymorphs of hen egg-white lysozyme crystals. It was found that in a number of cases the growth of one of the two faces was blocked, whereas the opposite one was not slowed down. To explain our results we propose a self-poisoning mechanism based on solvent-induced adsorption of misorientated lysozyme molecules on the inhibited faces. This mechanism can also prevent some proteins from forming crystals at all.

3.1 Introduction

X-ray diffraction remains the most important method to solve the 3D structure of biological macromolecules. The success of this method depends on whether protein crystals can be grown and if so, on their quality. To understand the factors responsible for this, knowledge of the mechanisms involving the formation of protein crystals is important. A model system for investigations into protein crystal growth mechanisms is the enzyme hen egg-white lysozyme (HEWL). Like many proteins[1], this protein can crystallise in various crystal structures depending on the crystallising agent and temperature, a phenomenon called polymorphism*. Lysozyme crystals grown from a sodium chloride solution have either the well-known tetragonal $P4_32_12$ structure or the orthorhombic $P2_12_12_1$ structure (at higher temperatures), while crystals grown from a sodium thiocyanate solution are monoclinic $P2_1$. HEWL crystals growing in a sodium nitrate solution can either turn out as monoclinic $P2_1$ or triclinic $P1$ (fig. 3.1). The difference in crystal structure also shows up

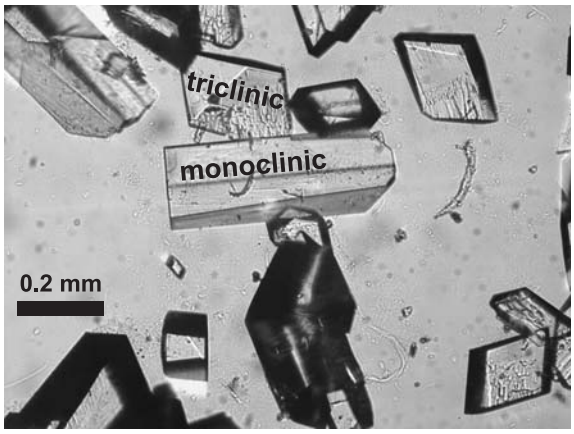

Figure 3.1: Monoclinic and triclinic HEWL crystals coexisting in a HEWL/ $NaNO_3$ / NaOAc / HOAc solution.

*The various possible crystal structures of lysozyme are not polymorphs in the strictest sense of the word, because the salts are incorporated in the crystal and thus induce different compositions

in the crystal morphology, or outer shape, of the crystals. An often observed feature of the monoclinic polymorph of lysozyme is an asymmetry in the crystal habit between the two opposite top faces of the rod shaped crystals (see figure 3.1 and refs. [2–5]).

A polar morphology, i.e. a crystal habit lacking inversion symmetry, is the result of opposite faces (hkl) and $(\bar{h}\bar{k}\bar{l})$ growing at a different rate, called polar growth, or unidirectional growth. A classical example is the growth of α-resorcinol, which shows polar growth from solution as well as from the vapour phase[6, 7]. A prerequisite for polar growth is a crystal structure in which the pair of opposite faces are not related by a symmetry operator of the crystal's point group. Typically, this is likely to occur for molecules which do not have inversion symmetry, like α-resorcinol and also proteins which, being build of only left-handed amino-acids, by definition do not have inversion symmetry. The Hartman-Perdok theory[11], relating growth rate to attachment energy, does not provide for difference in growth rates of opposite faces. Explanations can be found in surface-solvent interactions[8], presence of impurities[9, 10], and self-poisoning mechanisms[12].

Here we present a study on the formation of polar morphologies in protein crystal growth. By using optical microscopy, we compare growth rates of opposite crystal faces for the tetragonal, monoclinic and triclinic form of hen egg-white lysozyme crystals. Based on our experiments and literature data on lysozyme anion binding sites we propose a self-poisoning mechanism by misoriented lysozyme molecules adsorbed on one of the opposite polar surfaces, which leads to a reduction or complete arrest of its growth. A complete blocking of opposite faces offers a possible explanation for the fact that some proteins do not form crystals at all.

3.2 Experimental methods

Chemicals of analytical grade were used in this study. A buffer stock solution of sodium acetate and acetic acid was made in deionised water (>15 MΩcm) to result in a 0.05 M NaOAc/HOAc solution of pH 4.5. HEWL from Sigma-

Aldrich (lot nr. 094K1454) was used as source material for crystal growth after purification by dialysis (MWCO 8 kDa) in buffer solution. NaCl, $NaNO_3$ and NaSCN stock solutions were also prepared in buffer solution. Lysozyme, salt and buffer solutions were filtered over a 0.2 μm membrane (Schleicher & Schuell), and mixed with each other in the appropriate proportions just prior to the growth experiments. For experiments on the effects of impurities ultra-pure lysozyme (99.99%, Mol Logics Inc., Japan) was used without further treatment in filtered buffer and salt solutions.

Growth solutions were inserted in a cell consisting of an X-ring in between two microscope cover glasses and sealed by vacuum grease to prevent evaporation. The internal dimensions of the growth cell are a radius of 7 mm and a height of 1.8 mm, comprising a volume of 270 μl. Crystals were either nucleated in the cell or seeded into it. For the experiment, the growth cell was placed on a temperature-controlled stage with a hole of 6 mm in diameter to allow for transmission optical microscopy, while stabilising the temperature within a 0.3 ^{o}C margin.

Images were acquired by in-situ optical transmission microscopy in combination with a CCD camera. A combination of image processing software (ImagePro Plus [13]) and Matlab[14] was used to determine crystal side face displacement from the images. Using a macro in ImagePro Plus, image intensity line profiles perpendicular to the edges of the crystal top face, which correspond to the crystal side faces of interest, were taken from subsequent micrographs. In the intensity profiles these crystal surfaces show up as a sharp decrease in intensity with respect to the bright background. A script in Matlab is used to detect these decreases automatically for the series of line profiles, and thus the position of the surfaces with respect to the fixed image frame of the image as function of time.

3.3 Results & Discussion

3.3.1 Tetragonal lysozyme

Figure 3.2a shows a tetragonal lysozyme crystal growing from a 40 mg/ml HEWL, 0.685 M NaCl[†], 0.05 M NaOAc/HOAc solution at 21 ^{o}C. The crystal is viewed upon one of its {110} faces, with the *c*-axis parallel to the longer crystal direction. The aspect ratio of tetragonal lysozyme crystals changes with supersaturation[15], changing from plate-like at high to elongated crystals at low supersaturation. Curved lines inside the crystal are just visible, which indicate the different growth sectors of the crystal[4]. The point group of the tetragonal crystal structure of HEWL is 422. Because this point group has a four-fold axis along the *c*-axis and two-fold axes along the *a*- and *b*-axis, the four {110} faces are symmetrically equivalent as well as the eight {101} faces. As a result, the crystal growth rates are equal in opposite {110} directions (fig. 3.2b) and nearly equal in opposite {101} directions. The slight difference in the latter case is due to the presence of crystals outside the field of view influencing the nutrient supply to one of the two {101} faces. A difference in the number of dislocations outcropping at the surface may also account for this difference.

3.3.2 Monoclinic lysozyme

Monoclinic lysozyme crystals can be grown from sodium nitrate solutions and sodium thiocyanate solutions. Literature shows a slight difference in cell parameters for these two lysozyme-salt complexes (e.g. Protein Data Bank[16] entries 1HF4, using $NaNO_3$, and 1LCN, using NaSCN), as do the morphologies of the crystals (figs. 3.3a and b). In both cases the point group symmetry is 2. Figure 3.3a shows a series of images of a monoclinic lysozyme crystal growing in a 15 mg/ml HEWL, 0.2 M $NaNO_3$ 0.05 M NaOAc/HOAc solution at 20 ^oC. The crystal shows the typical polar morphology of the monoclinic polymorph. In the positive *b*-direction, as determined by Hondoh et al.[2], the (010) face is properly faceted. In the opposite direction the crystal has

[†]0.685 M NaCl = 4% w/v NaCl.

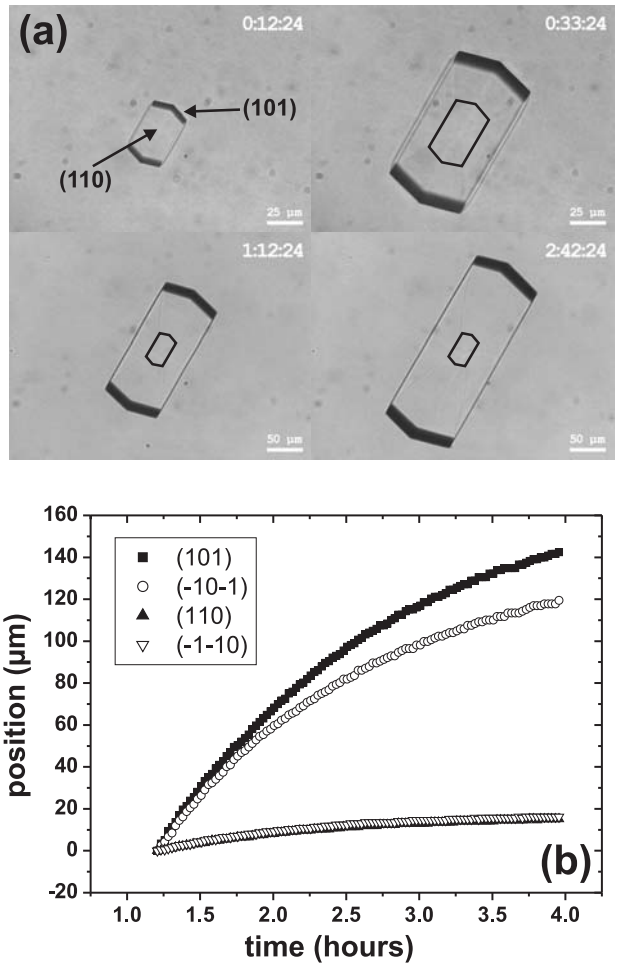

Figure 3.2: (a) Series of optical time-lapse images of a tetragonal lysozyme crystal growing in a 40 mg/ml HEWL, 4% w/v NaCl, 0.05 M NaOAc/HOAc solution of pH 4.5 at 21°C. Note that in the first two pictures the scale bar indicates 25 μm, and in the last two it indicates 50 μm. The crystal size of the first picture is represented in the other pictures by black lines. (b) Crystal surface position of the {110} and {101} surfaces of the crystal in (a). Zero indicates the position of the surfaces in the first image of the series.

a rounded surface which does not show any growth. As this rounded surface does not grow it is not kinetically roughened as suggested by Ref. [2], but it is round as a result of growth blockage over a range of directions containing a negative b component. Since the average orientation is $[0\bar{1}0]$, we shall indicate this set of faces $(h\bar{k}l)$ as $(0\bar{1}0)$.

Similar to the monoclinic $NaNO_3$-lysozyme crystal, the NaSCN-lysozyme crystal exhibits polar growth along the b-axis. Figure 3.3b shows a series of images of a monoclinic HEWL crystal growing in a 10 mg/ml HEWL, 0.1 M NaSCN, 0.05 M NaOAc/HOAc solution with the ultra-pure lysozyme, at 18 ^{o}C. We assume the similarity in crystal structure and morphology also to hold for the orientation of the polar faces of the thiocyanate-grown monoclinic polymorph, and thus identify the blocked and rounded face to be -on the average- the $(0\bar{1}0)$ face. New crystals preferably nucleate and grow out at this side. In comparison to the experiment with $NaNO_3$, the NaSCN solution has a higher supersaturation ($\Delta\mu/kT = 1.1$ versus $\Delta\mu/kT = 0.76$, with $\Delta\mu/kT = \ln(c/c_{\mathrm{eq}})$) and both the (010) and the side faces grow faster. Figure 3.4 shows the crystal surface position for the crystals in the thiocyanate and the nitrate experiments. The side faces show little asymmetry, while the (010) and $(0\bar{1}0)$ faces show one to two orders of magnitude difference in growth rate. This agrees with the point group 2 of the crystals for which the side faces $(h0l)$ and $\bar{h}0\bar{l}$) are symmetrically equivalent, in contrast to the opposite (010) and $(0\bar{1}0)$ pair. Experiments using lysozyme from Sigma, less pure than that used for the experiment shown in figure 3.3b, gave similar results. From this, we conclude that heterogeneous impurities cannot be responsible for the polar growth of monoclinic HEWL crystals.

3.3.3 Triclinic lysozyme

The triclinic polymorph of the lysozyme-nitrate system can be grown by first inducing nucleation of both the triclinic and the monoclinic polymorph by lowering the temperature. After a raise in temperature the monoclinic polymorph will dissolve while the stable triclinic polymorph remains[17, 18]. A triclinic lysozyme crystal was removed from the solution and seeded into a fresh growth

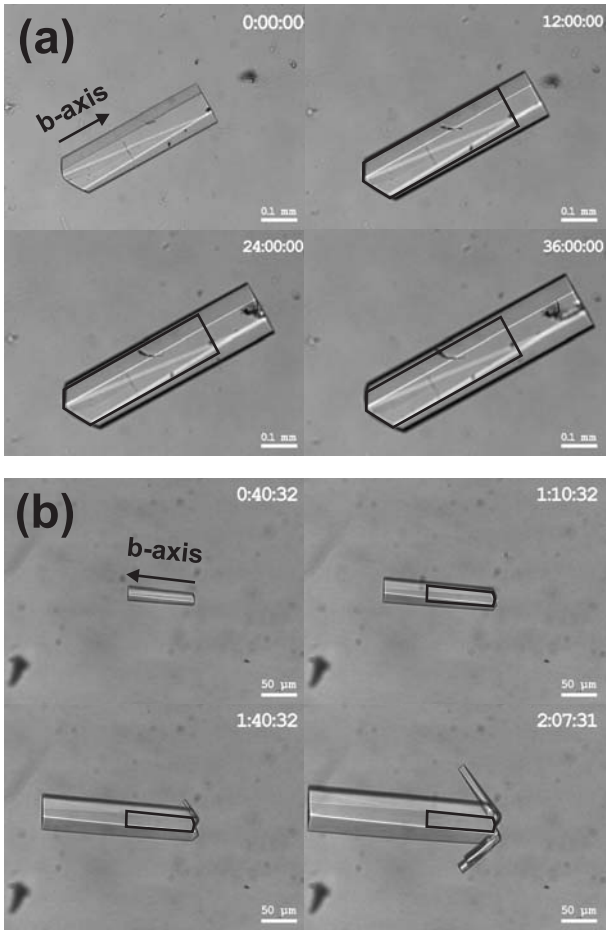

Figure 3.3: (a) Series of optical time-lapse images of a monoclinic lysozyme crystal growing in a 15 mg/ml HEWL, 0.2 M $NaNO_3$ 0.05 M NaOAc/HOAc solution of pH 4.5 at 20 ^oC. The black lines are a guide to the eye indicating the size of the crystal in the first image of the series. Time indicated in the upper right corners is in hours. (b) Series of optical time-lapse images of a monoclinic lysozyme crystal growing from in a 10 mg/ml HEWL, 0.1 M NaSCN, 0.05 M NaOAc/HOAc solution, at 18 ^{o}C, using ultra-pure lysozyme (99.99%). The black lines indicate the size of the crystal in the first image for comparison.

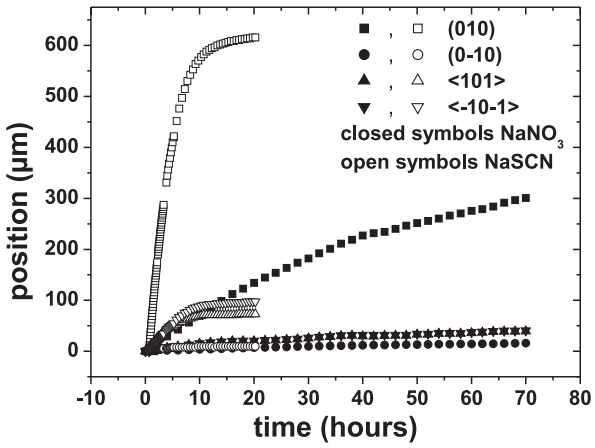

Figure 3.4: Crystal surface position for the (010) and the $(0\bar{1}0)$ surfaces of the monoclinic crystals in figure 3.3a (closed symbols), and figure 3.3b (open symbols).

solution of 5 mg/ml HEWL, 0.2 M $NaNO_3$ and 0.05 M NaOAc/HOAc. Figure 3.5a shows a time-lapse series of micrographs of the triclinic crystal exhibiting polar growth at 20 ^{o}C, at a supersaturation $\Delta\mu/kT$ of 1.1. Both directions of the crystal in the image plane show asymmetry in the growth rate (fig. 3.5b). The faces of one pair both show growth, the fastest about five times faster than the slowest; for the second pair one face is blocked in its growth. The growth rate of the third, out-of-plane direction cannot be determined from these images. In contrast to the polar surface of the monoclinic polymorph, all polar faces are properly faceted. New crystals often nucleate at the blocked face, which is similar to the monoclinic crystals grown from the NaSCN solutions.

3.3.4 Comparing lysozyme polymorphs

Polar growth, i.e. a difference in growth of opposite faces (hkl) never occurs if the point group of the crystal is centrosymmetric. Proteins are chiral growth units, so the crystal structure of these macromolecules will never contain an inversion centre or a (glide) mirror plane. So, protein crystals are prone to

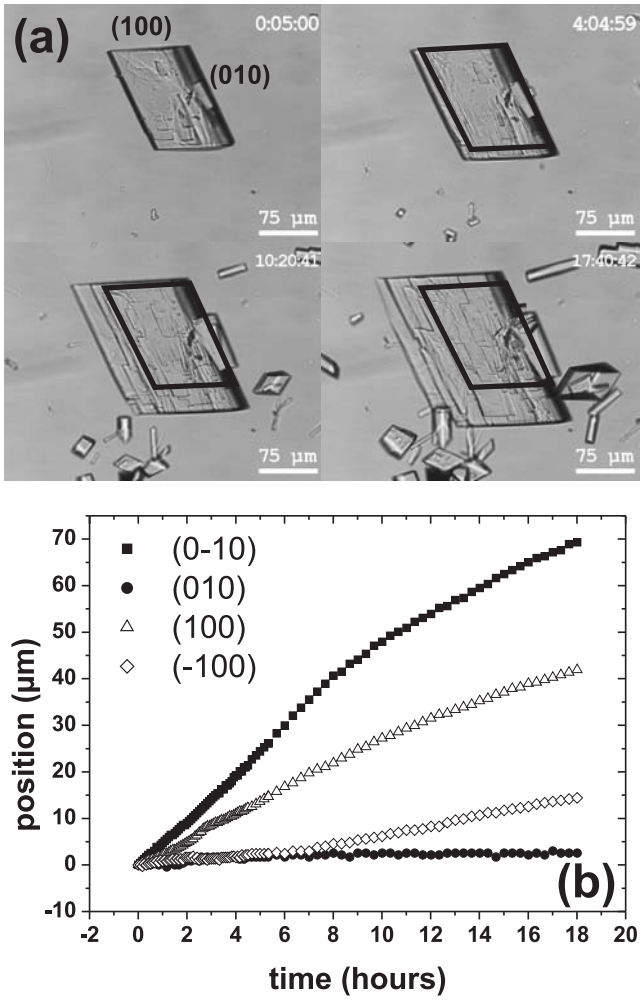

Figure 3.5: Series of optical time-lapse images of a triclinic lysozyme crystal growing in a 5 mg/ml HEWL, 0.2 M $NaNO_3$ and 0.05 M NaOAc/HOAc solution of pH 4.5 at 20 ^{o}C. The black lines in subsequent figures indicate the crystal size of the first image. The crystal exhibits polar growth in two crystallographic directions. Time indicated in the upper right corner is in hours. (b) Crystal surface position for the crystal in (a) showing quantitatively the polar growth of the {100} and {010} faces.

polar growth. For point groups with high symmetry it is more likely that opposite faces are linked by symmetry. For tetragonal lysozyme crystals with point group 422 the opposite faces of the forms {101} and {110}, which determine the morphology of the crystals, are symmetrically equivalent. Therefore, no polar growth is expected to occur, as is observed. For the monoclinic crystals with point group 2 only the side face pairs $(h0l)$ and $(\bar{h}0\bar{l})$ are related by symmetry. The top faces (010) and $(0\bar{1}0)$ are different, which leads to the observed polar growth along the b-axis. The point group of the triclinic crystals is 1, that is, they lack symmetry and all possible pairs of opposite faces (hkl) and $(\bar{h}\bar{k}\bar{l})$ are not symmetry related. Here polar growth can occur for all directions.

Often, an asymmetry in growth rate is caused by the presence of impurities in the solution. If these impurities preferably adsorb onto a specific crystal surface[9], this surface is slowed down or blocked for further growth[19]. In both "normal" and extra-pure solutions, the monoclinic polymorph grown from NaSCN solutions showed a growing (010) and a stationary $(0\bar{1}0)$ face. From this, we conclude that the presence of impurities are not a prerequisite for polar growth of lysozyme crystals.

The correlation between space group and number of polar growth directions suggest that the polar growth is an intrinsic property of the crystal structure. When we regard the interactions between lysozyme molecules using the macrobond concept as introduced by Hondoh et al.[2] and Matsuura et al.[20] there is no difference in a bond from a molecule A to a molecule B and vice versa. Thus, these bonds do not force a preferential direction for growth and also do not include solvent effects. Therefore, polar growth can not be explained from a simple point of view, only considering bulk bonds between adjacent growth units in the crystal.

The origin of the polar growth may be found in the interactions of the crystal surface with the solvent or with lysozyme molecules in the solvent. For crystals of small organic molecules, interactions with the solvent can result in a surface-bound layer of solvent molecules which prohibit the attachment of the crystal growth units[10, 21]. For proteins, the size difference between

the growth units and the solvent molecules, water molecules and ions, is very large, and it does not seem feasible that these block the attachment of a protein molecule. Experiment shows that for the monoclinic case polar growth is independent of the choice of the anion (i.e. NO_3^- or SCN^-). In fact, the presence of water or ions is even necessary to form a crystalline contact between molecules[22]. However, the solvent molecules can change the surface charge distribution by binding to the lysozyme molecule, and as such change the characteristics of the crystal surface.

For polar growth to occur the molecular structure of the opposite (hkl) and $(\bar{h}\bar{k}\bar{l})$ surfaces must differ. Figure 3.6a shows the orientation of the lysozyme molecule in the (001) and $(00\bar{1})$ surfaces of the triclinic polymorph, with the basic residues depicted in light grey and the acidic residues in dark grey. All directions show a different local composition of basic and acidic residues. These residues lead to a difference in charge density distribution over the molecule surface, which again leads to a difference in water and anion binding. Since these bindings form the interactions between the different molecules in the crystals a difference in growth rate in opposite directions seems feasible. The same conclusion can be drawn for the monoclinic polymorphs. Figure 3.6b shows a different local composition of residues for the (010) and $(0\bar{1}0)$ surfaces, but the other faces $(h0l)$ and $(\bar{h}0\bar{l})$ are identical.

No detectable growth was observed for the $[0\bar{1}0]$ directions of the monoclinic crystals as well as for one of the (010) faces of the triclinic crystals. Since impurities and small molecules can not explain this arrest in growth, we suggest that blocking is caused by wrongly oriented protein molecules covering the surface. It is expected that protein molecules adsorbed on the crystal surface can have several orientations, different from the correct one, which are still energetically favourable. If the surface is covered by a large number or a complete layer of such misoriented HEWL molecules, then no new layers can develop on top of it and crystal growth is blocked. A similar self-poisoning of crystal growth has also been proposed to explain the polar growth of the steroid crystal 7αMNa[12]. A self-poisoning mechanism due to misorientated protein molecules on the crystal surfaces may also explain the difficulty in

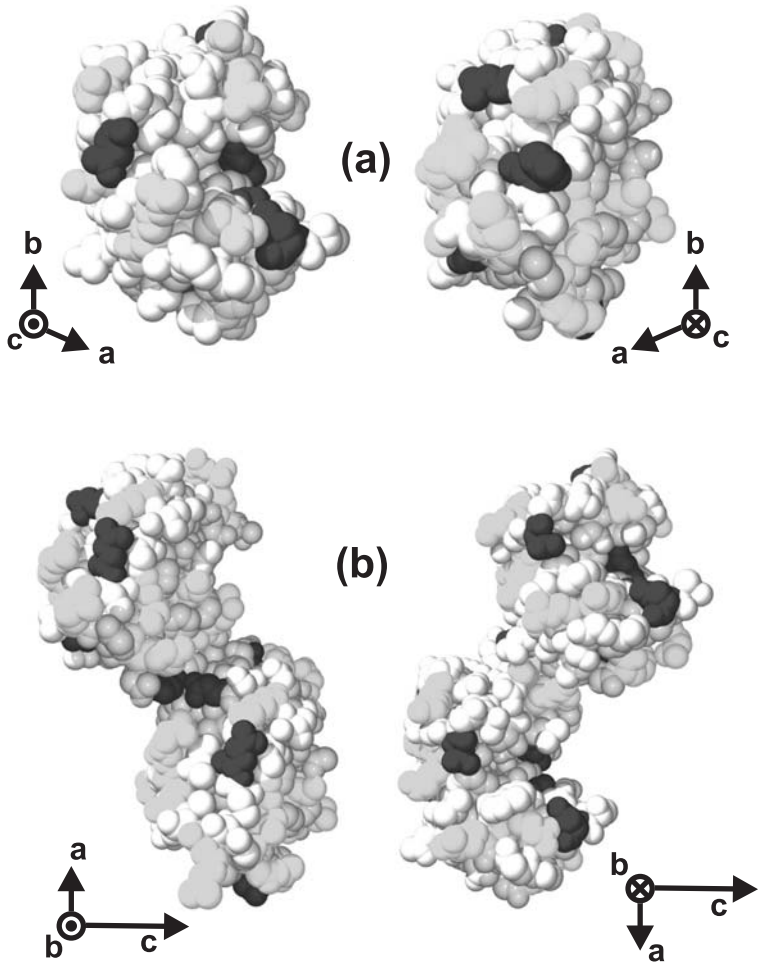

Figure 3.6: (a) Schematic representation of a hen egg-white lysozyme molecule viewed upon as if in the triclinic (001) (left) and (00$\bar{1}$) (right) surfaces. For this image structural data taken from the Protein Data Bank[16] (code 4LZT) was used. Dark grey colored atoms indicate acidic residues and light grey colored atoms indicate basic residues. (b) Schematic representation of the two lysozyme molecules in the asymmetric unit of the monoclinic structure, viewed upon its (010) face (left) and (0$\bar{1}$0) face (right), (PDB code 1HF4).

Figure 3.7: Clusters of monoclinic crystals in a 10 mg/ml, 0.1 M NaSCN, 0.05 M NaOAc/HOAc solution of pH 4.5 at 15 ^{o}C. The large crystal was seeded into the solution. The scale bar indicates 50 μm.

crystallisation of many proteins, because when the opposite faces are both blocked, growth of the crystal cannot occur.

Secondary nucleation often occurs at the rounded, blocked side of the monoclinic crystals grown from thiocyanate solutions. The formation of secondary crystallites was also encountered on the blocked (010) face of the triclinic crystals. This nucleation is partly the result of a higher supersaturation as the crystal does not grow and so does not lower the solute concentration at the blocked side. Further, in contrast to the growing parts of the crystals, sub-micron crystallites sedimented onto the surface[23] are not grown-in and develop into larger sized crystals. In addition misoriented HEWL molecules on the $(0\bar{1}\ 0)$ faces of monoclinic crystals might stimulate secondary heterogeneous nucleation, which results in clusters of crystals with the $+\vec{b}$ direction pointing outward, as often observed experimentally (figure 3.7).

3.4 Conclusion

Growth rates of crystal surfaces (hkl) and their opposites $(\bar{h}\bar{k}\bar{l})$ were investigated for four polymorphs of hen egg-white lysozyme. Crystals were found to grow polar in directions in which the opposite surfaces are not linked by symmetry operations. Therefore, the tetragonal $P4_32_12$ polymorph bounded by {110} and {101} faces cannot exhibit polar growth, whereas the monoclinic $P2_1$ structure can and does show polar growth along the b-axis for crystals grown from NaSCN and $NaNO_3$ solutions. The triclinic $P1$ polymorph, which does not have any symmetry related surfaces, showed polar growth in the two observable directions. For the monoclinic and triclinic crystals one of the polar faces is almost completely blocked in growth. Experiments using ultra-pure lysozyme showed that the presence of impurities is not a prerequisite for polar growth of lysozyme crystals. A simple view only considering bulk bonds between growth units can neither explain a polar morphology. We propose that self-poisoning induced by misoriented lysozyme molecules with an energetically favourable orientation on the crystal surface reduces or blocks growth and thus plays a major role in the polar growth of lysozyme crystals. Self-poisoning may also play an important role in the difficulties in crystallisation of many proteins, as crystal growth cannot occur if opposite faces both suffer from such a mechanism. The tuning of crystallisation conditions could then be viewed as the tuning of interactions to avoid self-poisoning.

Acknowledgements

The authors would like to thank dr. H. Meekes for stimulating discussions on polar growth, and Mirjam Theelen for performing preliminary experiments.

References

[1] Carugo, O.; Argos, P. *Protein Science* **1997,** *6,* 2261-2263.

[2] Hondoh, H.; Sazaki, G.; Miyashita, S.; Durbin, S.; Nakajima, K.; Matsuura, Y. *Crystal Growth & Design* **2001,** *1,* 327-332.

[3] Jones, W.; Wiencek, J.; Darcy, P. *Journal of Crystal Growth* **2001,** *232,* 221-228.

[4] Vidal, O.; Robert, M.; Arnoux, B.; Capelle, B. *Journal of Crystal Growth* **1999,** *196,* 559-571.

[5] Rashkovich, L.; Gvozdev, N.; Sil'nikova, M.; Chernov, A. *Crystallography Reports* **2002,** *47,* 859-866.

[6] Weissbuch, I.; Leiserowitz, L.; Lahav, M. *Crystal Growth & Design* **2006,** *6,* 625-628.

[7] Srinivasan, K.; Sherwood, J. *Crystal Growth & Design* **2005,** *5,* 1359-1370.

[8] Bisker-Leib, V.; Doherty, M. *Crystal Growth & Design* **2001,** *1,* 455-461.

[9] Orme, C.; Noy, A.; Wierzbicki, A.; McBride, M.; Grantham, M.; Teng, H.; Dove, P.; DeYoreo, J. *Nature* **2001,** *411,* 775-779.

[10] Weissbuch, I.; Popovitz-Biro, R.; Lahav, M.; Leiserowitz, L. *Acta Crystallographica* **1995,** *B51,* 115-148.

[11] Hartman, P.; Perdok, W. *Acta Crystallographica* **1955,** *8,* 49-52.

[12] Stoica, C.; Tinnemans, P.; Meekes, H.; van Enckevort, W.; Vlieg, E. *Crystal Growth & Design* **2006,** *6,* 1311-1317.

[13] ImagePro Plus 4.5, MediaCybernetics.

[14] MATLAB 6.5 (Release 13), The MathWorks Inc.

[15] Durbin, S.; Feher, G. *Journal of Crystal Growth* **1991,** *110,* 41-51.

[16] Berman, H.; Westbrook, J.; Feng, Z.; Gilliland, G.; Bhat, T.; Weissig, H.; Shindyalov, I.; Bourne, P. *Nucleic Acids Research* **2000,** *28,* 235-242.

[17] Legrand, L.; Ries-Kautt, M.; Robert, M. *Acta Crystallographica* **2002,** *D58,* 1564-1567.

[18] Heijna, M.; van den Dungen, P.; van Enckevort, W.; Vlieg, E. *Crystal Growth & Design* **2006,** *6,* 1206-1213.

[19] Plomp, M.; McPherson, A.; Malkin, A. *Proteins* **2003,** *50,* 486-495.

[20] Matsuura, Y.; Chernov, A. *Acta Crystallographica* **2003,** *59,* 1347-1356.

[21] Stoica, C.; Verwer, P.; Meekes, H.; van Hoof, P.; Kaspersen, F.; Vlieg, E. *Crystal Growth & Design* **2004,** *4,* 765-768.

[22] Frey, M.; Genovesio-Taverne, J.; Fontecilla-Camps, J. *Journal of Physics D: Applied Physics* **1991,** *24,* 105-110.

[23] Kuznetsov, Y.; A.J. Malkin, A. M. *Journal of Crystal Growth* **1999,** *196,* 489-502.