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The Effects of Impurities on Protein Crystal Growth and Nucleation: A Preliminary Study

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Introduction

Kubota and Mullin (1995) devised a simple model to account for the effects of impurities on crystal growth of small inorganic and organic molecules in aqueous solutions. Experimentally, the relative step velocity and crystal growth of these molecules asymptotically approach zero or non-zero values with increasing concentrations of impurities. Alternatively, the step velocity and crystal growth can linearly approach zero as the impurity concentration increases. The Kubota-Mullin model assumes that the impurity exhibits Langmuirian adsorption onto the crystal surface. Decreases in step velocities and subsequent growth rates are related to the fractional coverage (θ) of the crystal surface by adsorbed impurities; $\theta = Kx / (1+Kx)$, x=mole fraction of impurity in solution. In the presence of impurities, the relative step velocity, V/Vo, and the relative growth rate of a crystal face, G/Go, are proposed to conform to the following equations (Kubota, N. & Mullin, J.W.).

$$V/Vo \cong G/Go = 1 - \alpha\theta$$

The adsorption of impurity is assumed to be rapid and in quasi-equilibrium with the crystal surface sites available. When the value of α, an effectiveness factor, is one the growth will asymptotically approach zero with increasing concentrations of impurity. At values less than one, growth approaches a non-zero value asymptotically. When α is much greater than one, there will be a linear relationship between impurity concentration and growth rates. Kubota and Mullin expect α to decrease with increasing supersaturation and shrinking size of a two dimensional nucleus.

It is expected that impurity effects on protein crystal growth will exhibit behavior similar to that of impurities in small molecule growth. A number of proteins were added to purified chicken egg white lysozyme the effect on crystal nucleation and growth assessed.

Materials and Methods

Chicken egg-white lysozyme from Sigma (CEWL) was repurified by cation exchange chromatography and recrystallized as previously described (Forsythe, Ewing & Pusey). Turkey egg white lysozyme (TEWL), conalbumin, bovine ribonuclease A and thaumatin from Sigma and bovine serum albumin (BSA) from ICN Biochemicals were used without further purification and were added to CEWL as 'impurities'. All proteins were dialysed against distilled water followed by dialysis against 0.1 M sodium acetate, pH 4.6. CEWL and impurities were mixed in varying proportions. The CEWL solutions were mixed with buffer and 20% NaCl to a final NaCl concentration of 5% (w/v). Twenty microliters of the solution was placed in the well of a 24 well (6 X 4) crystalling plate and placed in an incubator at 20°C. The volume of the crystallizing solution did not change over time (batch crystallization) and CEWL concentration decreased over time as crystals formed. The first column of four wells in the crystallization plate contained no impurity and served as the control. Each column of the crystallizing plate contained four wells and at least one column was used for each initial CEWL concentration and impurity level. The impurity concentration is reported as % of total protein (w/w). Initial CEWL concentration was 15, 21 or 30 mg/ml and saturation concentration at 20°C, 5% NaCl, pH 4.6 is 1.8 mg/ml. Impurity level varied as follows: TEWL 0, 8.8, 14, 18, 22, and 26%, and 0, 4.5, 7, 10, 12.5, and 15%; conalbumin 0, 19, 32, 41, 48 and 54% and 0, 5, 11, 15, 19, and 23%; ribonuclease A 0, 6, 11, 16, 21, and 24%; BSA 0, 18, 31, 40, 47, and 52%; thaumatin 0, 15, 26, 35, 41, and 47% of total protein.

The crystallizing dishes were incubated for two to four weeks. Images of each well were taken and the number of crystals in each well counted. For plates containing TEWL the length of each crystal in the 101 and the 110 was measured only when both lengths were visible for an individual crystal. An aspect ratio was defined as the crystal length in the 101 direction divided by the crystal length in the 110 direction.

The aspect ratios and number of crystals in wells containing added impurity were compared to controls on the same crystallizing plate using a one way analysis of variance (ANOVA) followed by the Tukey multiple comparison test for normally distributed data. For data which was not normally distributed, a Kruskall-Wallis one way ANOVA on ranks followed by Dunn's multiple comparison test was performed. A multiple comparison test was performed only when significant differences were indicated by an ANOVA using SigmaStat software by Jandell.

Results

Turkey egg-white lysozyme (TEWL). There is an apparent change in morphology of CEWL crystals grown in the presence of TEWL. As the relative amount of TEWL increases, CEWL crystals become more elongated in the 101 direction. This effect is more pronounced in samples with an initial concentration of CEWL of 15 or 21 mg/ml than in samples with an initial CEWL concentration of 30 mg/ml (compare slopes of lines in figure 1). The aspect ratio, defined as the ratio of the crystal length in the 101 direction divided by the length in the 110 direction, is plotted as a function of % TEWL of total initial protein in figure 1. The number of CEWL crystals in wells with TEWL did not differ significantly from the number of crystals in control wells containing no TEWL for all initial concentrations of CEWL.

The aspect ratio of crystals in wells with added TEWL is compared to crystals in control wells with no TEWL on the same crystallizing plate using an analysis of variance. The data for each initial concentration of CEWL are from two separate experiments. For one data set with an initial CEWL concentration of 30 mg/ml, only the highest concentration of TEWL (26%) produced significant differences in the aspect ratio when compared to the control wells. A second experiment with lower TEWL concentrations (4.5%, 7.3%, 9.8%, 12.5%, 14.8%) produced crystals with aspect ratios that differed significantly from controls.

At an initial CEWL concentration of 21 mg/ml, all wells with added TEWL resulted in crystals with aspect ratios significantly larger than the control crystals except crystals grown in wells containing 7% TEWL. At an initial concentration of 15 mg/ml crystals grown in 10%, 15%, 18%, and 26% TEWL resulted in crystals with significantly larger aspect ratios compared with control crystals.

Conalbumin. Nucleation was inhibited at an initial concentration of CEWL of 15 mg/ml at very high levels of conalbumin (32% of total protein). At 19% conalbumin the total number of crystals formed did not differ significantly from the controls containing no added conalbumin. At initial CEWL concentration of 21 mg/ml, the results were unclear. In one set of experiments conalbumin concentrations of 11%, 15%, 19% and 23% resulted in formation of significantly more crystals than the controls. In another set of experiments, a greater number of crystals formed when total protein consisted of 48 or 54% conalbumin, but not in wells containing 19 to 41% conalbumin. At the highest initial concentration of CEWL (30 mg/ml), only very high concentrations of conalbumin (greater than 48%) resulted in significantly more crystals when compared with controls.

The morphology of CEWL crystals formed in the presence of conalbumin at any level (from 6% to 54%) was effected at all initial concentrations of CEWL (15, 21 and 31 mg/ml). Crystal edges became less sharp with increasingly greater additions of conalbumin (figure 3). Crystals were submitted for evaluation by X-ray diffraction It is unclear how the diffraction quality of the crystals is effected by addition of conalbumin.

In further experiments with conalbumin, a 220 microliter quantity of solution with a relative supersaturation of 6 (bulk concentration divided by equilibrium solubility of CEWL at 20°C, 5%NaCl, pH 4.6) was placed in the well of a tissue culture plate. Controls contained no conalbumin. Five replicates each with 5, 10 and 20% conalbumin were either seeded with two microliters of seed solution of relative supersaturation of 35 (incubated for 2 minutes at 20°C) or left unseeded. The plates were incubated at 20°C for approximately two weeks and crystals were counted and measured. There were significantly fewer crystals (no crystals formed) in unseeded wells with 20% added conalbumin in comparison to the controls wells. There were no other

significant differences in the number of crystals in seeded or unseeded wells at any level of conalbumin. Crystals in wells with added conalbumin were significantly smaller than crystals in wells with no added conalbumin.

Figure 1. The effect of TEWL on the aspect ratio of CEWL crystals. Symbols represent the average value of four replicates and one sided error bars represent one standard deviation. The lines are a straight line fit to the data for each initial concentration of CEWL and are drawn only to indicate the general trend of the data with increasing amounts of added TEWL.

Bovine serum albumin (BSA). Addition of BSA to CEWL solutions produced no evident effect on nucleation of CEWL in solutions containing an initial concentration of 15 or 30 mg/ml. At an initial concentration of 21 mg/ml significantly more crystals were produced in wells containing 18% or 40 % conalbumin, but not in wells with 31, 47 or 52% conalbumin.

There was a change in morphology of CEWL crystals grown in the presence of BSA. It appeared that growth of the 101 face was inhibited with increasing proportions of BSA.

Bovine ribonuclease A. Addition of ribonuclease A to CEWL resulted in a significant increase in the number of CEWL crystals compared to controls for all initial concentrations of CEWL tested. At an initial CEWL concentration of 15 mg/ml the number of crystals in a 20 microliter drop of solution was 7.0±2.9. At 6% to 24% ribonuclease the average number of crystals in a 20 microliter drop varied from 114 to 262, with numbers of crystals generally increasing with increasing concentration of ribonuclease A (table 1).

Table 1. Average number of crystals formed in 20 microliter drop with initial CEWL concentration of 15 mg/ml.

% Ribonuclease A (w/w total protein)	Mean number of crystals in 20 microliter drop	Standard deviation of number of crystals
0	7.0	2.9
6	127	12
11	114	19
16	149	28
21	197	17
24	262	57

At initial CEWL concentrations of 21 and 31 mg/ml the average number of crystals in wells containing no ribonuclease was 17±2.4 and 30±1.2 respectively. Crystals in wells containing ribonuclease were too numerous to count, but are estimated to range from 300 to 1000. All crystals exhibited the normal tetragonal morphology of CEWL crystals.

Thaumatin. At an initial CEWL concentration of 15 mg/ml there was no significant difference in the number of crystals formed in wells containing 6 to 11% thaumatin. At higher thaumatin concentration, few (17% thaumatin) or no ((22 to 28% thaumatin) crystals formed. At initial CEWL concentrations of 21 and 30 mg/ml an average of 20 and 44 crystals formed in control wells. In all wells with added thaumatin, an average of 300 to 1,000 crystals formed. All crystals appeared to exhibit the normal CEWL tetragonal morphology.

Discussion and Conclusions

Protein impurity effects on CEWL crystal growth and morphology are specific and show a concentration dependency. TEWL, a protein with high sequence homology to CEWL, inhibits growth of the 110 face. Conalbumin and BSA cause the morphology of CEWL to change. BSA appears to inhibit growth of the 101 face. Conalbumin has been shown to inhibit growth of the 101 face (Judge *et. al.*).

CEWL and TEWL are both composed of 129 amino acids and differ by seven amino acids. Apparent inhibition of growth of the 110 face of CEWL by TEWL results in elongation of the crystals grown in the presence of TEWL. This effect is more pronounced at lower

supersaturation (initial CEWL concentration of 15 mg/ml and 21 mg/ml) than at higher supersaturation (30 mg/ml). This behavior is consistent with the effects predicted by the Kubota and Mullin model and with the behavior of impurities in small molecule growth. The lowest concentration of TEWL used was 5%. Because the crystals are grown in batch wells, the concentration of CEWL will decease as crystals grow. If TEWL is rejected by the crystal the %TEWL (w/w) will increase as the crystallization proceeds and the inhibitory effect on growth may increase over time. Additions of TEWL did not appear to effect nucleation rates.

Conalbumin is a protein which is also present in chicken egg-white and is therefore frequently found in chicken egg-white lysozyme. Conalbumin has a reported PI between 6.1 and a molecular weight of 76 kilodaltons and is structurally dissimilar to CEWL. In studies by Judge *et. al.* conalbumin was found to inhibit growth of the 101 face of CEWL crystals at low supersaturation (14 mg/ml at 18°C where solubility was 1.3 mg/ml) and at high concentration of impurity (30%). At higher concentrations of CEWL, growth rates were not effected by this level of conalbumin. As predicted by the Kubota - Mullin model, supersaturation can modify the inhibitory effect of impurities. Growth was not inhibited with 10% conalbumin at the CEWL concentrations studied by Judge *et. al.*

Addition of large quantities of BSA (18% or greater), did not have a large effect on nucleation of CEWL crystals but appeared to inhibit growth of the 101 face. BSA has a PI of about 4.8 and a molecular weight of 60 kilodaltons.

The effects of impurity additions on nucleation of crystals was not clear. Nucleation was found to both increase and decrease with addition of impurities, depending on supersaturation, % impurity, and the specific protein.

The preliminary studies outlined here indicate that conalbumin effects nucleation of CEWL crystals. This affect appears to be modified by supersaturation. At the lowest concentrations of CEWL examined, 11 mg/ml and 15 mg/ml, nucleation of crystals was completely inhibited with 20% and 32% added conalbumin, respectively. At higher initial concentrations (21 and 31 mg/ml CEWL), nucleation is not inhibited and in some cases a more crystals are formed at high impurity concentration. Edges of CEWL crystals were roughened by addition of any amount of conalbumin at all initial CEWL concentrations examined here.

Bovine ribonuclease A dramatically increased nucleation of CEWL at all initial concentrations of CEWL (15, 21, and 31 mg/ml). Ribonuclease has a PI of 9.3 and a molecular weight of 13.7 kilodaltons. With additions of 6% to 24% ribonuclease, the average number of crystals formed in a 20 microliter drop increased from 7 to 30 to a range of 114 to several hundred. This is in contrast to the results of Abergel *et al.* who demonstrated that nucleation of TEWL was inhibited by addition of ribonuclease.

Addition of thaumatin (PI of 12 and molecular weight of 22 kilodaltons) to solutions inhibited nucleation of CEWL crystals at low initial concentration of CEWL and resulted in increased nucleation at higher initial concentrations of CEWL.

A summary of the effect of impurities on nucleation and growth of CEWL crystals is found in table 2. Only TEWL is homologous with CEWL. Of the proteins which show little sequence homology to CEWL, those with high molecular weight and PI below 7 modified the morphology of CEWL crystals. Dissimilar proteins of relatively high PI and low molecular effected nucleation, but appeared to have little effect on crystal morphology.

Table 2. Summary of effect of protein impurities on nucleation and morphology of CEWL crystals.

Protein	PI (Molecular weight, kd)	Effect nucleation	Effect Growth / Morphology
CEWL	10.7 (14.3)		
TEWL	(14.3)	no	yes
Conalbumin	6.1 (76)	yes (increase & decrease)	yes
BSA	4.8 (60)	no	yes
Ribonuclease A	9.3 (13.7)	yes (increase)	no
Thaumatin	12 (22)	yes (increase & decrease)	no

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