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Growth of (101) faces of tetragonal lysozyme crystals: measured growth-rate trends

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Previous extensive measurements of the growth rates of the (110) face of tetragonal lysozyme crystals have shown unexpected dependencies on the supersaturation. In this study, similar growth-rate measurements were performed for the (101) faces of the crystals. The data show a similar dependence on the supersaturation, becoming appreciable only at high supersaturations, reaching a maximum value and then decreasing. The (101) growth rates are larger at low supersaturations than the (110) growth rates under the same conditions and are smaller at high supersaturations. These trends suggest that the growth mechanism of the (101) face is similar to that of the (110) face: both processes involve the addition of multimeric growth units formed in solution, but the average size of the units for the (101) face is likely to be smaller than for the (110) face.

1. Introduction

Recent investigations into the growth kinetics and crystal structure of tetragonal lysozyme crystals have greatly increased our understanding of the growth mechanisms of the crystal faces. This is particularly true of the (110) face. Electron microscopy and atomic force microscopy (AFM) studies have shown that the growth steps on this face were always at least two molecules high and that its surface corresponded to the plane containing the 2_1 crystallographic axes (Durbin & Feher, 1990; Durbin & Carlson, 1992; Konnert *et al.*, 1994; Li, Perozzo *et al.*, 1999). These results, combined with a periodic bond-chain analysis of the tetragonal lysozyme crystal structure, indicated that the crystallizing unit on this face was at least a tetramer corresponding to the 4_3 crystallographic axes (Nadarajah & Pusey, 1996).

In earlier studies, we made extensive measurements of the macroscopic growth rates of the (110) face of tetragonal lysozyme at different temperatures and protein concentrations (Nadarajah *et al.*, 1995). These growth rates showed an unexpected dependence on the supersaturation. Relatively large supersaturations were required to achieve appreciable growth rates. When the supersaturations were increased further, the growth rates reached a maximum before decreasing. Lowering the supersaturations caused the growth rates to decrease asymptotically to zero. This general behavior persisted when the pH, salt and protein concentrations were changed, producing growth rate *versus* supersaturation curves of consistent overall shape throughout.

We had proposed that growth by a multimeric growth unit, formed in solution by self-association reactions, could explain the above experimental observations. Such reactions are a

consequence of the nucleation process, and the low-level oligomers formed are intermediates on the pathway to a critical nucleus. These reactions must then persist in the growth solution as a function of the solute concentration. We developed a mathematical model of the growth process, assuming a reversible sequence of doubling self-association reactions at equilibrium, leading to the formation of dimers, tetramers, octamers and 16-mers in solution (Li *et al.*, 1995; Nadarajah *et al.*, 1997). The growth unit comes from this mixture and attaches to the crystal face by dislocation or two-dimensional nucleation growth mechanisms.

The best fits to the measured (110) growth-rate data with this model were obtained when the growth unit was assumed to be octamers. This was in agreement with predictions from crystallographic analyses, which suggested that the growth unit must at least be a helical tetramer corresponding to the 4_3 axes (Nadarajah & Pusey, 1996; Strom & Bennema, 1997*a*,*b*). The enthalpies for the self-association process obtained from these fits also agreed well with those obtained from the solubility data, further suggesting the validity of this mechanism for the (110) face involving octamer growth units. Still further support comes from dialysis kinetics studies, where the predicted concentrations of monomers and dimers were found to agree with the measured concentrations (Wilson *et al.*, 1996), and by studies which have shown the presence of lower and higher order clusters in lysozyme solutions under crystallization conditions (Pusey, 1991; Wilson & Pusey, 1992; Behlke & Knespel, 1996; Minezaki *et al.*, 1996).

Unlike for the (110) face, extensive measurements have not been completed for the (101) face. The limited measurements of the growth rate for this face have indicated similarities and deviations when compared with those for the (110) face. For example, it has been observed that the (101) faces grow faster at lower supersaturations, but are exceeded by the growth rates of the (110) faces at higher supersaturations (Durbin & Feher, 1986; Monaco & Rosenberger, 1993; Forsythe & Pusey, 1994). However, these data are inadequate for the systematic determination or verification of the growth mechanisms for the (101) face. The goal of this study was to collect such data at different solution conditions, but with particular emphasis on temperature and protein concentration. The data can then be analyzed to determine the growth mechanism of this face.

2. Materials and methods

Chicken egg-white lysozyme was purchased from Sigma and repurified as previously described (Ewing *et al.*, 1996). The final protein solutions were maintained at the desired pH with 0.1 *M* acetate buffer. Protein concentrations in these solutions were determined by UV absorbance (Aune & Tanford, 1969).

Growth-rate measurements were made using a computer-controlled microscopy system as previously described (Pusey, 1993). The system consists of a horizontally mounted video-microscopy system focused on a temperature-controlled glass growth cell through an ultra-long working distance microscope objective. The growth cell is in turn mounted on a pair of orthogonal translation stages and the microscope mounted on a third stage, giving the *X*, *Y* and *Z* axes of motion, respectively. The movements of the translation stages are controlled by a computer. The signal from the video camera is processed by a circuit which, again through control of the computer, can be used to pre-select individual video scan lines and trigger their digitization by a high-speed digital oscilloscope board located within the computer. A series of crystals of suitable size (10–30 µm), which have grown attached to the cell walls and are located such that the (101) faces are parallel to the viewing axis, are selected by scanning through the growth cell and their locations are stored in the computer memory. For each crystal, a pair of horizontal video scan lines are selected which cross the faces to be measured. The digitized scan lines are used to determine where the line crosses the crystal face. These give a pair of coordinates (points) which, together with the measured angles of the faces to the horizontal scan lines (slope) and video-microscopy calibration factors (vertical distance per scan line and horizontal distance per digitized point), are used to calculate the distance between the faces.

Measurement runs are initiated after set-up by injection of a freshly prepared growth solution into the cell followed by initiating the measurement sequence. Seed crystals are freshly nucleated in the growth cell prior to each rate determination and dissolved by a distilled water wash at the conclusion of the measurement run; thus, each crystal is only used for one growth-rate determination. The sequence of events during a measurement cycle are as follows. The computer sets the translation stages to the coordinates for the first crystal, then digitizes the selected video lines for that crystal and determines its size. The coordinates for the second crystal are then set and the process is repeated for each crystal in the sequence. This process is repeated 12–15 times, with the time interval between cycles being adjusted by the operator to account for the different growth rates. Employing this approach, the (101) faces of 4–20 suitably aligned tetragonal crystals were selected for the growth-rate determination at each set of conditions, the number being smaller the greater the supersaturation. For each run, the growth system was maintained at a constant temperature throughout while the seed crystals were nucleated and the growth-rate measurements were made.

In typical inorganic crystal-growth experiments, to ensure that the measured bulk protein concentration and the interfacial concentration are the same, the solutions are stirred vigorously during growth. However, since lysozyme crystal growth is usually not mass-transport limited, this assumption can be made even in unstirred solutions. Nevertheless, as a further precaution growth-rate measurements were made on relatively small crystals and each experiment was halted well before the growth rates started to decrease owing to protein depletion in the bulk solution. Starting seed-crystal sizes typically ranged from 10 to 30 µm, ensuring that interfacial and bulk protein concentrations were very close and that convective flows were minimized (Pusey & Naumann, 1986; Pusey *et al.*, 1986). The duration of each experimental run ranged from less than an hour to a few days, which was large enough to verify that the growth rates remained constant

throughout this period. The experimental runs where the growth rates did change over time, owing to solution depletion caused by excessive nucleation, were rejected. The total number of these discarded runs was small and represented less than 2% of all collected data.

Three widely differing growth conditions for tetragonal lysozyme crystals were chosen and the growth rates were measured by varying the temperature and solution protein concentration at each condition. These conditions were (i) pH 4.0, 5%(*w*/*v*) NaCl, (ii) pH 4.6, 3%(*w*/*v*) NaCl and (iii) pH 5.0, 5%(*w*/*v*) NaCl, all in 0.1 *M* sodium acetate (NaAc) buffer, and are the same as those employed in our earlier study of the (110) face (Nadarajah *et al.*, 1995). Depending on the conditions, the protein concentration C ranged from 1 to 40 mg ml^{-1} and the temperature from 277 to 293 K. Some of the pH 4.0 data have been published previously (Forsythe & Pusey, 1994). The growth rates obtained were plotted against the supersaturation σ [where $\sigma = \ln(C/S)$] and against temperature. The lysozyme solubilities S were obtained from published values (Cacioppo & Pusey, 1991).

3. Results and discussion

The measured growth rates are plotted against the supersaturation for each of the three data sets and are shown in Figs. 1, 2 and 3. It can be seen that the growth-rate trends with supersaturation resemble those observed for the (110) face from our earlier studies, with similar shapes for each of the three data sets (Nadarajah *et al.*, 1995). When the supersaturation is lowered, the growth rates decrease asymptotically. With increasing supersaturations, the growth rates increase and reach a maximum value, after which they start decreasing. Additionally, as with the (110) face, a dead zone was not observed. As the supersaturation was decreased to very low values the growth rates decreased monotonically, but did not come to a halt before the saturation limit. Measurements were stopped in the range 10^{-5}–10^{-4} nm s^{-1} only because the measurement times become inordinately long.

These growth-rate trends are in agreement with those we have previously reported for the (101) face (Forsythe & Pusey, 1994). However, they differ somewhat from those observed in other studies (Monaco & Rosenberger, 1993; Vekilov *et al.*, 1993; Vekilov & Rosenberger, 1996). Those measurements were for a single protein and salt concentration and pH, namely 50 mg ml^{-1} lysozyme, 2.5% NaCl, pH 4.5 and 0.05 *M* NaAc buffer. The pronounced decreases in growth rates with increasing supersaturation which we report here were not observed in those studies. This can be attributed to the absence of data at very high supersaturations. However, other deviations require a different explanation.

We did not measure the growth rates of (101) faces at 2.5% NaCl, pH 4.5 and 0.05 *M* NaAc in this study, but we have made measurements on the (110) face at these conditions. They are

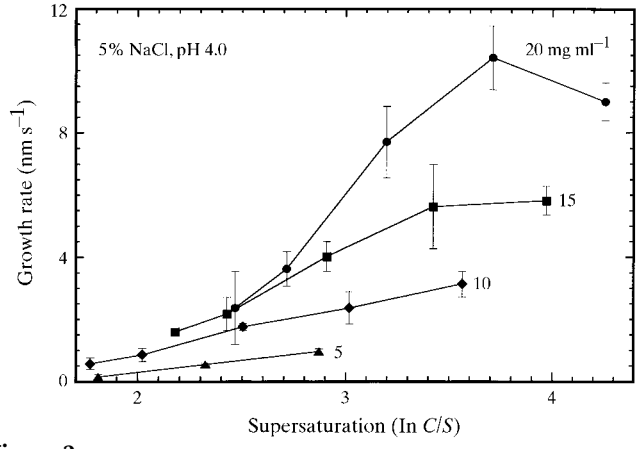

Figure 2
Measured growth rates of the (101) face of tetragonal lysozyme crystals at 5% NaCl, pH 4.0 and 0.1 *M* NaAc buffer for four protein-solution concentrations. The supersaturation was varied by changing the temperature from 277 to 295 K.

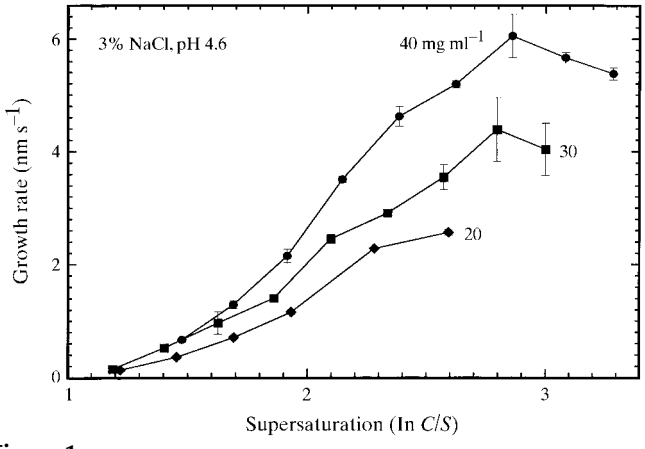

Figure 1
Measured growth rates of the (101) face of tetragonal lysozyme crystals at 3% NaCl, pH 4.6 and 0.1 *M* NaAc buffer for three protein-solution concentrations. The supersaturation was varied by changing the temperature from 277 to 295 K.

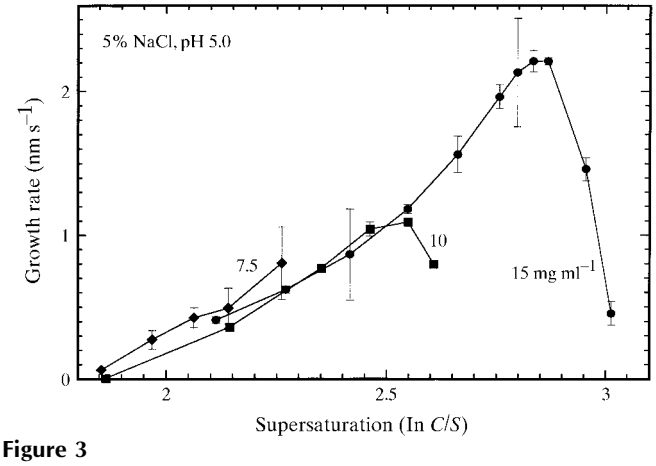

Figure 3
Measured growth rates of the (101) face of tetragonal lysozyme crystals at 5% NaCl, pH 5.0 and 0.1 *M* NaAc buffer for three protein-solution concentrations. The supersaturation was varied by changing the temperature from 277 to 295 K.

plotted in Fig. 4, along with (110) growth-rate measurements interpolated from Figs. 6 and 7 of Vekilov & Rosenberger (1996), which are representative of previous data. The solubility data for these conditions were obtained from published values (Rosenberger *et al.*, 1993). The similarity of the growth-rate trends for the two faces should enable us to infer the reasons for the difference in the (101) growth-rate data from comparisons using the (110) data.

The comparisons are complicated somewhat by the purity levels of the lysozyme solutions employed. Rosenberger and co-workers and many other investigators tend to employ protein solutions prepared directly from lysozyme purchased from various biochemical supply houses. As shown in Fig. 4, Vekilov & Rosenberger (1996) found a difference in crystal-growth rates with lysozyme purchased from Sigma and from Seikogaku. The primary difference between the proteins supplied is that the Seikogaku lysozyme was purified by crystallizing six times, while the Sigma lysozyme was crystallized only three times. Although they did perform further purification of the protein, they only report growth-step velocities with it and not growth rates. In contrast, all the commercially obtained protein in our study was extensively purified to $\geq$99.9% as described previously (Ewing *et al.*, 1996), resulting in protein solutions essentially free from all macromolecular impurities. The growth-rate data obtained with this protein were also highly reproducible, as shown by the relatively small variations in the multiple measurements made at each condition (see error bars in Figs. 1, 2, 3 and 4). Thus, the lower growth rates observed in earlier studies could have been the result of the impurity content of the lysozyme solutions.

The phase transition of lysozyme crystals from the tetragonal to the orthorhombic form is at least as important as the issue of protein purity. This temperature-induced transition is widely known (Berthou & Jollès, 1974) and was generally believed to occur at higher temperatures close to the physiological temperature. In more recent solubility studies of lysozyme, it was shown that the transition takes place at variable temperatures depending on the salt concentration and pH (Cacioppo *et al.*, 1991). For most conditions, such as the ones employed in our growth-rate measurements, the transition occurs between 297 and 308 K. However, at 2.5% NaCl, pH 4.5 and 0.05 *M* NaAc the transition from tetragonal to orthorhombic occurs at 293 K (Sazaki *et al.*, 1996). For a protein concentration of 50 mg ml^{-1}, this corresponds to a supersaturation σ = 1.65 as shown in Fig. 4.

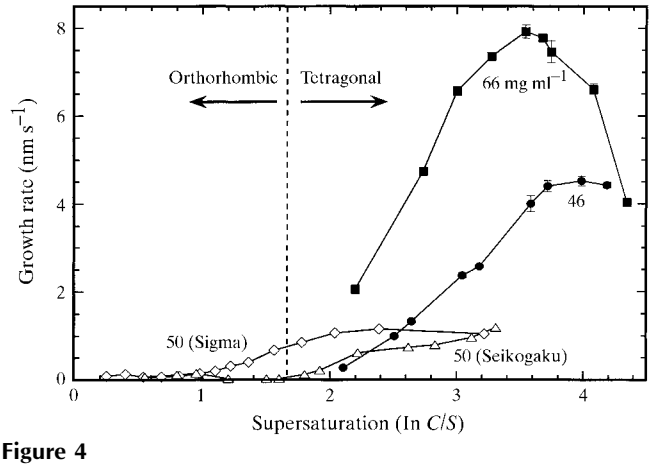

Figure 4
Comparison of measured growth rates of the (110) face at 2.5% NaCl, pH 4.5 and 0.05 *M* NaAc buffer. The solid symbols (■ and ●) are for purified lysozyme solutions at concentrations of 66 and 46 mg ml^{-1}, respectively, in this study. The open symbols (◇ and △) are for unpurified lysozyme solutions at a concentration of 50 mg ml^{-1} purchased from Sigma and Seikogaku, respectively, in the study of Vekilov & Rosenberger (1996). The dotted line is the supersaturation at 50 mg ml^{-1} (σ = 1.65), for the orthorhombic–tetragonal phase transition. The supersaturations are all calculated for tetragonal lysozyme solubility.

Fig. 4 shows that our measured (110) growth-rate data at 2.5% NaCl, pH 4.5 and 0.05 *M* NaAc follows similar trends as at the other conditions (Nadarajah *et al.*, 1995). It also resembles the (101) growth-rate trends measured in this study. However, all these measurements were made well below 293 K in the tetragonal phase region of lysozyme. In contrast, more than half the growth-rate measurements in earlier studies were made above 293 K. These measurements were all made on the same crystal by ramping the temperature up or down. Thus, growth-rate measurements were made while the conditions were changed back and forth across the tetragonal–orthorhombic phase transition. When measurements were performed in this manner, dead zones were observed for σ = 1.6 (Monaco & Rosenberger, 1993; Vekilov *et al.*, 1993; Vekilov & Rosenberger, 1996). This was attributed to the presence of impurities based on similar observations in inorganic crystal growth. However, the fact that the dead zones occur just below the phase transition to the orthorhombic phase at σ = 1.65 suggests that these two phenomena are related; the growth stoppage for the tetragonal crystals above 293 K could have been caused by orthorhombic crystal growth being favored at these conditions. Similarly, when the temperatures are ramped down from this region to the tetragonal-phase region below 293 K, growth rates may not return immediately to their normal value, leading to discrepancies in the measured values. If this is correct, it would explain the discrepancy between our growth-rate measurements and earlier ones.

Although the growth rates of lysozyme crystals are likely to be affected when the conditions are changed back and forth across the tetragonal–orthorhombic phase transition, the extent of any change and its mechanism is not clear. When the conditions are changed to the orthorhombic phase, tetragonal crystals slowly redissolve into solution and form orthorhombic crystals (Berthou & Jollès, 1974). Storing lysozyme solutions at orthorhombic phase conditions later affects the nucleation process for tetragonal crystals. Fewer crystals are nucleated in such solutions than in controls continuously stored under tetragonal conditions (Ewing *et al.*, 1996). However, such stored solutions do not seem to affect the growth rate of already nucleated tetragonal crystals. The growth and nucleation processes at the phase-transition region are complex issues which are beyond the scope of this study. Until the transition is better understood, it is best to avoid this region during growth studies.

When selecting the conditions at which growth-rate measurements are to be made, the above consideration must be kept in mind. In this study we have employed very low supersaturations while avoiding the tetragonal–orthorhombic transition. If the dead zones observed by others are an artifact of the phase transition, it would explain their absence in this study and our earlier study of the (110) face (Nadarajah *et al.*, 1995) under these conditions. This could have been noticed by others if they had collected data over a range of conditions rather than focusing on growth only at 2.5% NaCl, pH 4.5 and 0.05 *M* NaAc. The phase-transition temperature is usually not as low as 293 K and is easily avoided under other conditions. Comprehensive data would have also enabled the effects of macromolecular impurities (if any) to be separated from those arising from the phase transition.

The above comparisons also suggest the importance of comprehensive data collection of growth rates and of purifying the protein, regardless of the source, prior to crystal-growth studies. Given the complexity of the protein crystallization process, with the presence of multiple solid phases (such as crystals and amorphous precipitates) and multiple crystal forms (such as the orthorhombic and monoclinic forms), care must be exercised in interpreting the data. Moreover, there are considerable similarities between small and macromolecular crystal-growth processes. Thus, it is tempting to interpret protein crystal growth in terms of the better known growth processes of inorganic crystals, which are based on data collected over a narrow range of conditions while ignoring other phases. Analyses of the data collected over a wide range of conditions and taking into consideration other protein phases will provide a more complete view of the protein crystal-growth process. It will also more clearly distinguish the similarities and the differences between protein and inorganic crystal growth.

The similarity between the growth-rate trends for the (101) and the (110) faces of tetragonal lysozyme crystals is clearly seen when the growth rates are plotted against temperature as shown in Fig. 5. This similarity suggests that the growth mechanism for the (101) face is similar to that for the (110) face. As mentioned earlier, macroscopic modeling of the (110) growth rates indicated that growth proceeds by the formation of lysozyme clusters in solution followed by their attachment to the crystal face by dislocation or two-dimensional nucleation growth mechanisms. Octamer units corresponding to the 4_3 helices in the crystal structure were found to provide the best fit to the data (Li *et al.*, 1995; Wilson *et al.*, 1996; Nadarajah & Pusey, 1996; Nadarajah *et al.*, 1997). The formation of these units in the nutrient solution can be conveniently represented by a series of reversible doubling reactions given by

$$\text{monomer} \rightleftharpoons \text{dimer} \rightleftharpoons \text{tetramer} \rightleftharpoons \text{octamer} \rightleftharpoons \text{higher order } n\text{-mers},$$

although the actual pathway may be different. At fixed salt and buffer concentrations and pH, the relative populations of *n*-mers in the above equation are controlled by the total protein concentration in solution and the temperature. At low concentrations and high temperatures monomers and dimers will predominate, while octamer concentrations will be very low (Li *et al.*, 1995). When the total concentration is increased and the temperature decreased, the concentrations of smaller units, such as dimers and tetramers, would have increased, peaked and would now be decreasing, while the octamer concentration would be increasing. At still higher total concentrations and low temperatures the octamer concentration would also start decreasing after attaining a maximum, as still higher order growth units are formed. The (110) face growth-rate trends with the supersaturation or the tempera-

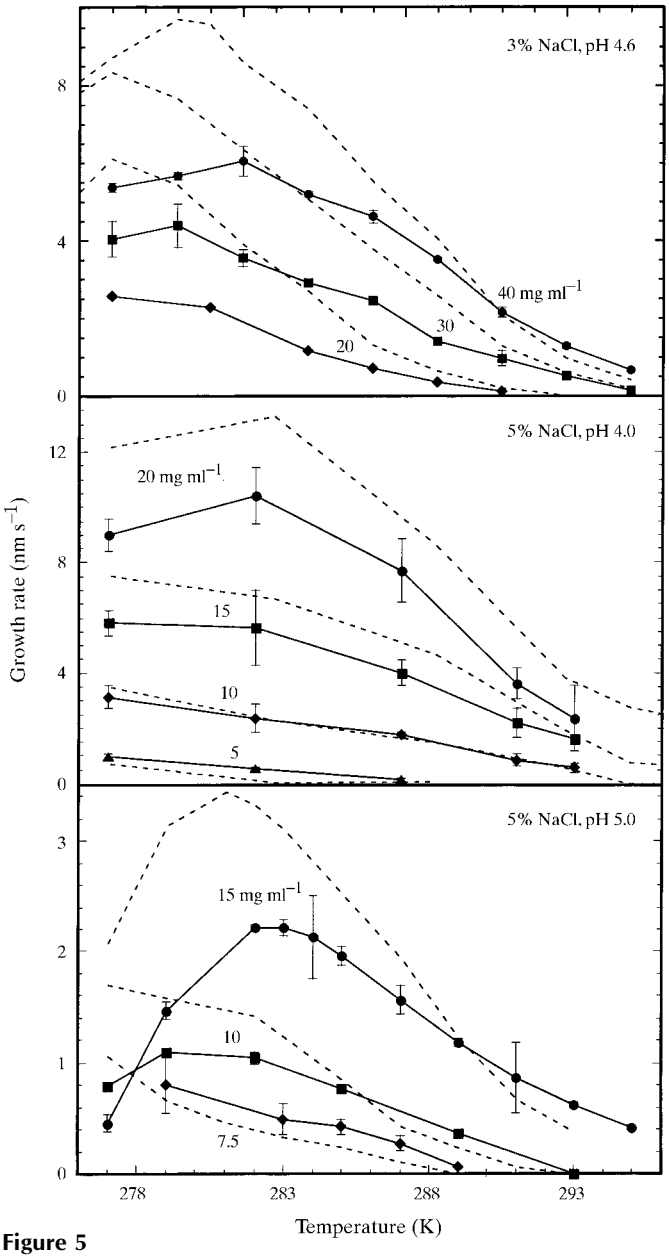

Figure 5
The solid lines show the measured growth-rate data at different conditions for the (101) face in Figs. 1, 2 and 3, but plotted against the temperature. The dotted lines show the growth-rate data under the same conditions for the (110) face published previously (Nadarajah *et al.*, 1995).

ture were shown to resemble those for the octamer concentration described above, which suggested that the growth was primarily driven by octamer growth units (Li *et al.*, 1995; Nadarajah *et al.*, 1997).

More recent studies employing high-resolution AFM techniques have shown that the molecular-growth process of the (110) face is even more complex (Li, Nadarajah *et al.*, 1999). Growth was shown to proceed by a variety of growth units corresponding to helices about the 4_3 axes, with tetramers the smallest observed unit. This means that the macroscopic models which assume growth proceeds by only one growth unit are only an average representation of the many different molecular-growth processes. Thus, for the (110) face the octamer unit represents the average size of all units participating in the growth process and not the sole growth unit.

Although the growth rates for the (101) face show trends similar to that for the (110) face, Fig. 5 clearly indicates that there are differences. For a given protein concentration, higher temperatures cause the (101) growth rates to be higher, while at lower temperatures the (110) growth rates dominate. Additionally, as the temperature decreases, the (101) growth rates reach a maximum value and begin decreasing before the rates for the (110) face. These observations suggest that the averaged growth unit for the (101) face is also a multimer, but one smaller than the octamer unit of the (110) face. Our earlier analysis of the crystal-packing arrangements of tetragonal lysozyme has indicated that the growth units for both faces must at least be a tetramer. Thus, we can expect the growth of the (101) face to proceed by many aggregate units with the tetramer as the minimum size. The average growth unit size can be expected to be smaller than an octamer, but larger than a tetramer.

4. Concluding remarks

The complexity of the protein crystallization process makes it imperative that growth-rate data be collected over a wide range of conditions and the analyses be performed while keeping in mind the presence of other protein phases. Additionally, caution is necessary in interpreting the more complex protein crystal-growth processes in terms of those for inorganic crystal growth based on superficial resemblance of growth-rate trends. Similarities do exist between the processes, such as growth by dislocation and two-dimensional nucleation. However, there are major differences as well, particularly in regard to the importance of solution-phase interactions to the growth process. These have been shown by our studies and by others, such as growth by aggregate units and three-dimensional nucleation growth (Malkin *et al.*, 1996*a*,*b*; Yip *et al.*, 1998).

The growth-rate measurements performed in this study suggest that the growth of the (101) face of tetragonal lysozyme crystals proceeds not only by the addition of multimeric growth units, but by units whose average size is smaller than that for the (110) face. The existence of different averaged growth units for the two faces explains the differences in the growth kinetics and morphology of these faces noted in earlier studies (Durbin & Feher, 1986; Monaco & Rosenberger, 1993; Forsythe & Pusey, 1994). The faster growth rates of the (101) faces at high temperatures or low supersaturations causes the crystals to have a needle-like shape, while at low temperatures or high supersaturations the faster growth rates of the (110) faces result in flat crystals. The minimum growth step height on the (110) face was found to be bimolecular, while that for the (101) face was only unimolecular (Durbin & Feher, 1990; Durbin & Carlson, 1992; Konnert *et al.*, 1994). Our related study to determine the molecular-growth mechanism of the (110) face also suggests growth by such multimeric units (Li, Nadarajah *et al.*, 1999).

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