Monomer Concentrations and Dimerization Constants in Crystallizing Lysozyme Solutions by Dialysis Kinetics

L. J. Wilson,* L. Adcock-Downey,# and M. L. Pusey§
*Department of Chemistry, East Tennessee State University, Johnson City, Tennessee 37614; #Mevatech Corporation, Huntsville, Alabama 35806; and §Biophysics Branch, NASA/Marshall Space Flight Center, Huntsville, Alabama 35812 USA

ABSTRACT Dialysis kinetics measurements have been made to study the effect of ionic strength on the dimerization of lysozyme in acidic solutions that lead to the growth of tetragonal lysozyme crystals. Using glutaraldehyde cross-linked dimers of lysozyme, we have determined that both monomers and dimers can escape from 25,000 molecular weight cutoff dialysis membranes with velocity constants of 5.1×10^{-7} and 1.9×10^{-7} s^{-1} for the monomer and dimer species, respectively. The flux from 25K MWCO membranes has been measured for lysozyme in pH 4.0 buffered solutions of 1, 3, 4, 5, and 7% NaCl over a wide range of protein concentrations. Assuming that dimerization is the first step in crystallization, a simple monomer to dimer equilibrium was used to model the flux rates. Dimerization constants calculated at low protein concentrations were 265, 750, 1212, and 7879 M^{-1} for 3, 4, 5, and 7% NaCl, respectively. These values indicate that dimerization increases with the ionic strength of the solution suggesting that aggregation is moderated by electrostatic interactions. At high protein concentrations and high supersaturation, the dimerization model does not describe the data well. However, the Li model that uses a pathway of monomer <-> dimer <-> tetramer <-> octamer <-> 16-mer fits the measured flux data remarkably well suggesting the presence of higher order aggregates in crystallizing solutions.

INTRODUCTION

Almost all proteins are crystallized with the aid of precipitating agents. These agents are useful in lowering the solubility of the protein allowing supersaturation to be achieved at practical protein concentrations. The most common precipitating agents are ionic salts. For lysozyme, many different salts can crystallize the protein resulting in at least five different crystal forms (Reis-Kautt and Ducruix, 1989). The use of NaCl as a precipitating agent for lysozyme can produce tetragonal or orthorhombic crystals depending on the pH and temperature. Very high concentrations of NaCl have been shown to precipitate lysozyme in a disordered manner. This suggests that the salt can mediate the protein-protein interactions and control the strength of the protein-protein bonds. Very strong attraction between protein molecules is thought to result in disordered or nonstructured aggregates (Skouri et al., 1992). The formation of protein crystals requires that the attraction between molecules be weak to moderate allowing time for formation of structured aggregates. Low order aggregates held together by such weak to moderate strength attractive forces are difficult to detect with traditional separation techniques such as size-exclusion chromatography. Also, the high salt conditions required to induce crystalline aggregates precludes use of native or denaturing gel electrophoresis.

Several groups have turned to quasi-elastic light scattering (QELS) to detect crystalline aggregates. For lysozyme, QELS results vary. Kam et al. (1985) used QELS as a predictor for the formation of crystals or precipitate assuming dimer formation as the first step in crystallization. Skouri et al. (1991) used QELS to study lysozyme interactions and found two distinct populations of aggregates. One population of soluble protein was assumed to be monomers and another group of large, presumably crystalline, particles grew larger with time. Georgalis et al. (1992) used photon correlation spectroscopy to study lysozyme and detected the presence of large precrystallization fractal clusters. Mikol et al. (1990) used QELS to study these solutions and suggested that crystal growth proceeded by attachment of monomers or small aggregates and that the critical nucleus consisted of only a few molecules. More recently, Muschol (1994) used QELS to measure the polydispersity of lysozyme solutions and was not able to detect aggregates in undersaturated solutions. One of the reasons that QELS has provided contradictory results is the inherent lack of sensitivity of the method. QELS gives an indirect measurement of the size distribution of species by interpreting mass changes from diffusivity measurements. A 50% decrease in the diffusivity corresponds to an eightfold increase in mass for spherical particles. Furthermore, it is difficult to detect aggregation in a polydisperse solution with QELS when the predominant species is the monomer and the remaining solute molecules are low order aggregates whose molar concentrations are decreasing with increasing size.

Recently, more sensitive techniques have been applied to lysozyme solutions. Boue et al. (1993) carried out small angle neutron scattering experiments that showed an increase in the average radius of gyration of molecules in high ionic strength solutions. They found the largest identifiable species in solution to be an octamer. Covalent cross-linking followed by SDS-PAGE separation has recently been used to detect aggregates of lysozyme in high ionic strength

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Address reprint requests to Dr. Lori J. Wilson, Chemistry Department, East Tennessee State University, Box 70695, Johnson City, TN 37614. Tel.: 423-929-6912; Fax: 423-929-5835; E-mail: wilsonl@etsu.-tn.edu.

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solutions with the largest identifiable species being a tetramer (Wang et al., 1996).

We used a dialysis kinetics technique to study the role of sodium chloride on the aggregation process of lysozyme. This technique measures the flux of protein molecules across a porous membrane. The flux is proportional to the size and diffusivity of the protein as well as the concentration difference across the membrane. Previous work with this technique used only two salt concentrations, 1 and 3% NaCl, to represent undersaturated and oversaturated lysozyme solutions, respectively (Wilson et al., 1993). We have expanded the salt concentration range to include solutions of high supersaturation and have used membranes permeable to monomers and dimers. Excellent agreement between our data and the 1 <-> 2 <-> 4 <-> 8 <-> 16 aggregation pathway proposed by Li et al. (1995) was found.

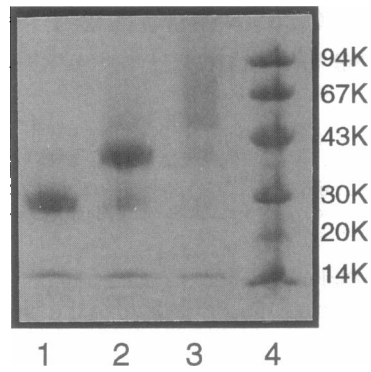

FIGURE 1 SDS-PAGE of fractions from size-exclusion column. Lane 1 contains 3 μg of purified dimer with a purity of 87% determined from densitometry. Lane 2 contains 3 μg of purified trimer found to be 77% pure by densitometry. Lane 3 contains higher molecular weight aggregates that are not well resolved. Lane 4 contains Pharmacia's low molecular weight standards as marked.

MATERIALS AND METHODS

Materials

Chicken egg white lysozyme was purchased from Calbiochem. All buffer components, except monobasic sodium phosphate, sodium chloride, and 70% glutaraldehyde (all from Fisher-Scientific Company, Boston, MA) were reagent grade from Sigma Chemical Company (St. Louis, MO). Cellulose ester dialysis tubing was from Spectrum Medical Industries (Houston, TX). Electrophoretic reagents, standards, and precast gels were from Pharmacia Biotech (Uppsala, Sweden).

Lysozyme was dissolved in and dialyzed against distilled water overnight to remove salts and buffers. It was then redialyzed against 1%(w/v) NaCl (0.1 M NaAc, pH 4.0). The dialysis buffer was saved and used to dilute the protein solution when preparing working solution of varying protein concentration. All working solutions were stored in a water bath at 20°C until used. For example, to prepare a protein working solution in 3% (w/v) NaCl (0.1 M NaAc, pH 4.0), equal volumes of the protein solution and 5% (w/v) NaCl (0.1 M NaAc, pH 4.0) were mixed. Lysozyme concentrations were determined using the absorption coefficient A (1%, 281.5 nm) = 26.4 (Aune and Tanford, 1969) on a varian DMS 200 UV-VIS spectrophotometer.

Cross-linking

Covalently cross-linked lysozyme dimers and trimers were prepared by the addition of 200 μl of 70% glutaraldehyde (aqueous) to 5 ml of 20 mg/ml lysozyme in 3% (w/v) NaCl (0.1 M NaAc, pH 4.0). After 5 min the reaction mixture was loaded onto a 115 × 2.5 cm size exclusion column of G-50 Sephadex running at 0.2 ml/min. Fractions were collected and analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) on Pharmacia's PhastSystem using the recommended protocols. After the initial chromatography step the dimer preparations were found to be 87% pure from densitometry (Fig. 1) using PhastImage (Pharmacia LKB Biotechnology AB). The samples were dialyzed against distilled water to remove salts and buffers and lyophilized for storage. Before use in the dialysis kinetics system the purified dimers were exhaustively dialyzed against 1% NaCl (0.1 M NaAc, pH 4.0) using a 15,000 molecular weight cutoff (MWCO) membrane to remove any monomeric lysozyme that was not separated on the size-exclusion column.

Dialysis kinetics

Although the dialysis kinetics apparatus has been described previously (Wilson et al., 1993), several modifications have been made. An obvious delay in the emergence of protein from the dialysis cell was seen during the first 2 to 15 min of each run. This delay time was thought to be due to two possible effects: 1) bulk solution flowing into the dialysis bag due to the osmotic effect of the protein or 2) difficulty of the relatively bulky protein in diffusing through the pores of the membrane. It was not anticipated that there would be any significant osmotic effect of the protein since the protein concentrations were 36 times less than the buffer concentration and 61 to 428 times less than the salt concentration both of which were present at equal concentration inside the dialysis cell and in the bulk solution. To be certain that no osmotic backflow of bulk solution into the dialysis cell was occurring, slight pressure was placed on the protein solution inside the cell (see Fig. 2). Further modifications were made to the cell to minimize the internal volume although maintaining the membrane surface area.

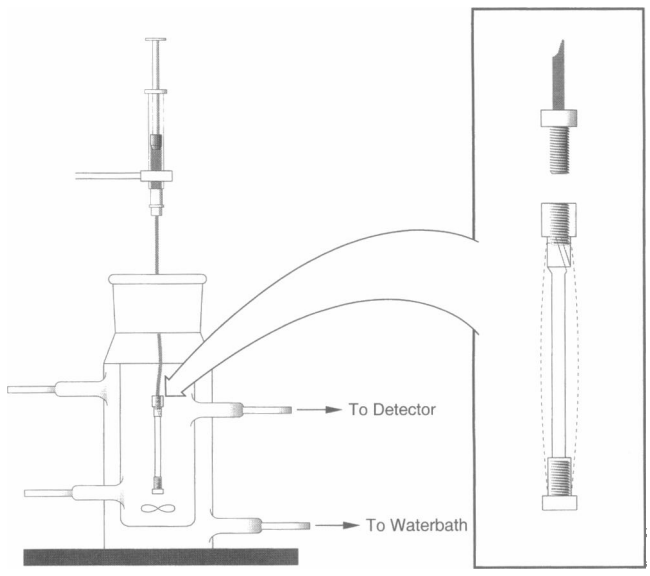

FIGURE 2 Dialysis kinetics system. A thermostated beaker (20°C) holds the protein cell and bulk solution. The bulk solution was stirred at 300 rpm and circulated through a UV/VIS detector. The protein was loaded into the dialysis membrane with a disposable 3-ml syringe and gentle pressure was applied and maintained with a clamp to counteract the osmotic backflow of bulk solution into the cell.

The rest of the system consisted of a thermostated beaker within which water was circulated by a circulating bath to maintain the temperature at 20°C. Approximately 115 ml of the buffered salt solution, also kept at 20°C, was placed in the beaker for each run. The bulk solution was circulated through an ISCO model 229 UV-VIS detector monitoring the absorbance at 281.5 nm. Although the external volume of 115 ml is large it provided sufficient sensitivity because of lysozyme's high absorbance at 281.5 nm. This volume would have to be reduced with proteins other than lysozyme to maximize the sensitivity of the technique. The output voltage of the detector was recorded using an NB-MIO-16X multi-purpose acquisition board in a Macintosh II cx computer running LabVIEW (National Instruments Corporation, Austin, TX). The output voltage of the detector was converted to protein concentration using the absorbtivity of lysozyme (Aune and Tanford, 1969). The flux of the protein was defined by the escape of protein molecules from the dialysis cell into the bulk solution as a function of time. The flux was determined by linear regression analysis of the graph of concentration of lysozyme in the bulk solution with time during the first 1000 to 2000 s. To test if there was any dilution of the protein during this time frame the total protein concentration was measured at the end of each run. It was found that the initial and final protein concentrations were equal relying on the *t*-test at the 95% confidence level. Furthermore, it was assumed that the minute amount of monomer removed from the cell would not result in any significant shift in the aggregation equilibrium.

Calibration of each membrane was done with monomers and cross-linked dimers of lysozyme. The flux of each species was measured as a function of its concentration. The slope of the calibration curve was defined as the velocity constant using the terminology of Hampe et al. (1982).

RESULTS

Characterization of dialysis membranes

Dialysis is a separation process that depends on the transport of solutes of different sizes across a porous barrier separating two liquids. The driving force of this process is the concentration gradient between the two sides when all other factors are held constant. Dialysis is typically used to separate species too large to transverse the membrane from those small enough to pass through it. Careful selection of membranes makes it possible to control which species can transverse the membrane.

Commercial membranes are characterized by their MWCO values that have been determined by the manufacturer. These values depend heavily on the solutes used for characterization and are not to be trusted for critical applications. It was, therefore, necessary to determine which substances are retained by the membrane. Because low ionic strength acidic solutions of lysozyme have been shown to be monomeric by light scattering (Bishop et al., 1992) and dialysis kinetics (Wilson and Pusey, 1993), we used these conditions to determine the monomer flux rate from 25,000 MWCO membranes. Fig. 3 *a* shows that the flux of lysozyme in 1% NaCl and pH 4.0 (0.1 M NaAc) varied linearly (R = 0.996) with the total concentration for the concentration range of 0 to 34 mg/ml with a slope or velocity constant of $5.1 \times 10^{-7}\ s^{-1}$.

Using purified covalently cross-linked dimers of lysozyme it was found that dimers were able to escape from a 25,000 MWCO membrane. The flux of the dimer was found to be linear (R = 0.982) with concentration in 1% NaCl and pH 4.0 (0.1 M NaAc) and is shown in Fig. 3 *b*. The velocity constant for the dimer was $1.9 \times 10^{-7}\ s^{-1}$ which is 37% lower than the velocity constant of monomeric lysozyme. Therefore, the total flux from a 25,000 MWCO membrane is described by:

$$f_{tot} = 5.1 \times 10^{-7}\ s^{-1}[M] + 1.9 \times 10^{-7}\ s^{-1}[D] \quad (1)$$

where f_{tot} is the measured flux from a 25,000 MWCO membrane, [M] is the molar monomer concentration and [D] is the molar dimer concentration.

Flux rate measurements

Once it was established that both monomers and dimers can penetrate the 25,000 MWCO membranes the flux of lysozyme was measured for salt concentrations of 3, 4, 5, and

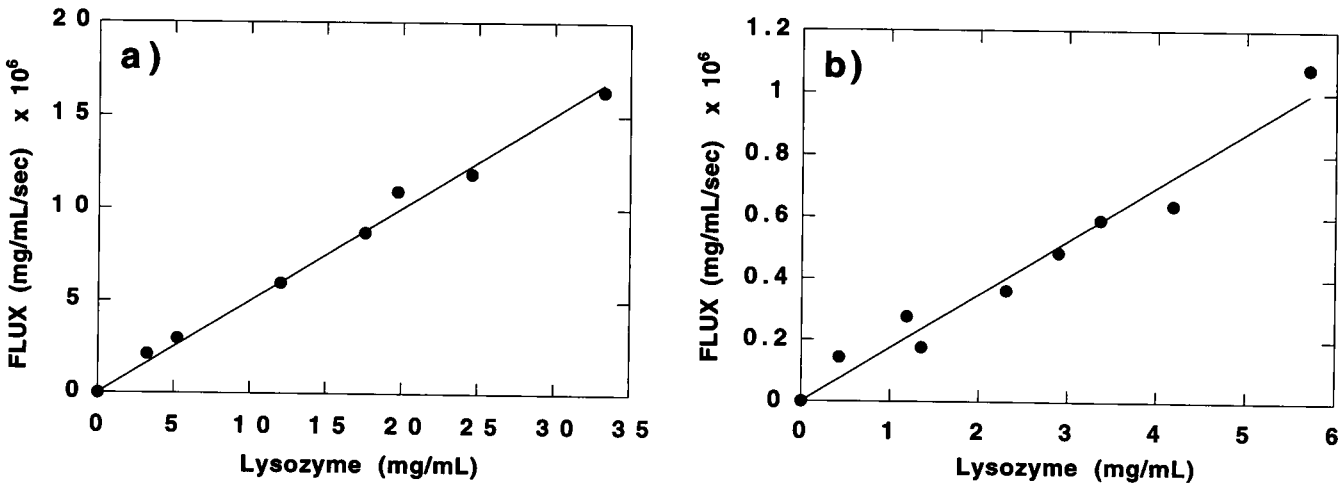

FIGURE 3 Calibration of a 25,000 MWCO membrane. *a*, Monomeric solutions gave a linear flux over the concentration range of 0–35 mg/ml. The slope of this plot was defined as the velocity constant and has a value of $5.1 \times 10^{-7}\ s^{-1}$ (R = 0.996). *b*, Purified covalently cross-linked dimer solutions were found to escape from a 25,000 MWCO membrane with a velocity constant of $1.9 \times 10^{-7}\ s^{-1}$ (R = 0.981) which is 37% less than the monomer velocity constant.

7% NaCl and pH 4.0 (0.1 M NaAc). Fig. 4 *a-d* shows how the flux varied with changes in the total concentration of lysozyme. Each point represents the average flux for three trials and the error bars are the SD for the three trials. The range of concentrations able to be studied decreased with increasing salt concentration. At high salt and protein concentrations, crystals formed inside the dialysis cell preventing an accurate measurement of the total lysozyme concentration. However, metastable solutions were obtained at lysozyme concentrations below 30, 23, 14, and 11 mg/ml for 3, 4, 5, and 7% NaCl, respectively. Above these concentrations crystals were clearly visible inside the dialysis cell within the 30 min it took to make a measurement. Below these concentrations no crystals were detected by visual inspection.

From Fig. 4 it is clear that in all cases the flux is linear with concentration at low protein concentration but deviates from linearity at higher protein concentration. This deviation from linearity occurs before the solubility concentration of lysozyme, C_{sat}, measured by Cacioppo and Pusey (1991) and marked on each graph. That a linear flux is detected in 1% NaCl up to 35 mg/ml but curvature is detected in higher salt solutions suggests aggregation and a subsequent decrease in the concentration of escapable units. The possibility that lysozyme is binding to the bag is ruled out because there is no detectable change in the protein concentration inside the bag. Furthermore, the initial flux is the same at all salt concentrations and low protein concentration suggesting that the salt concentration does not effect the proteins permeability though the membrane.

Calculation of dimerization constants

The solid curves of Fig. 4 *a–d* correspond to the calculated flux rate for a simple monomer to dimer equilibrium obtained from Eq. 1 where monomer and dimer concentrations were calculated using nonlinear least squares regression based on the following equilibrium:

$$2[M] \langle \rightleftharpoons \rangle [D]$$

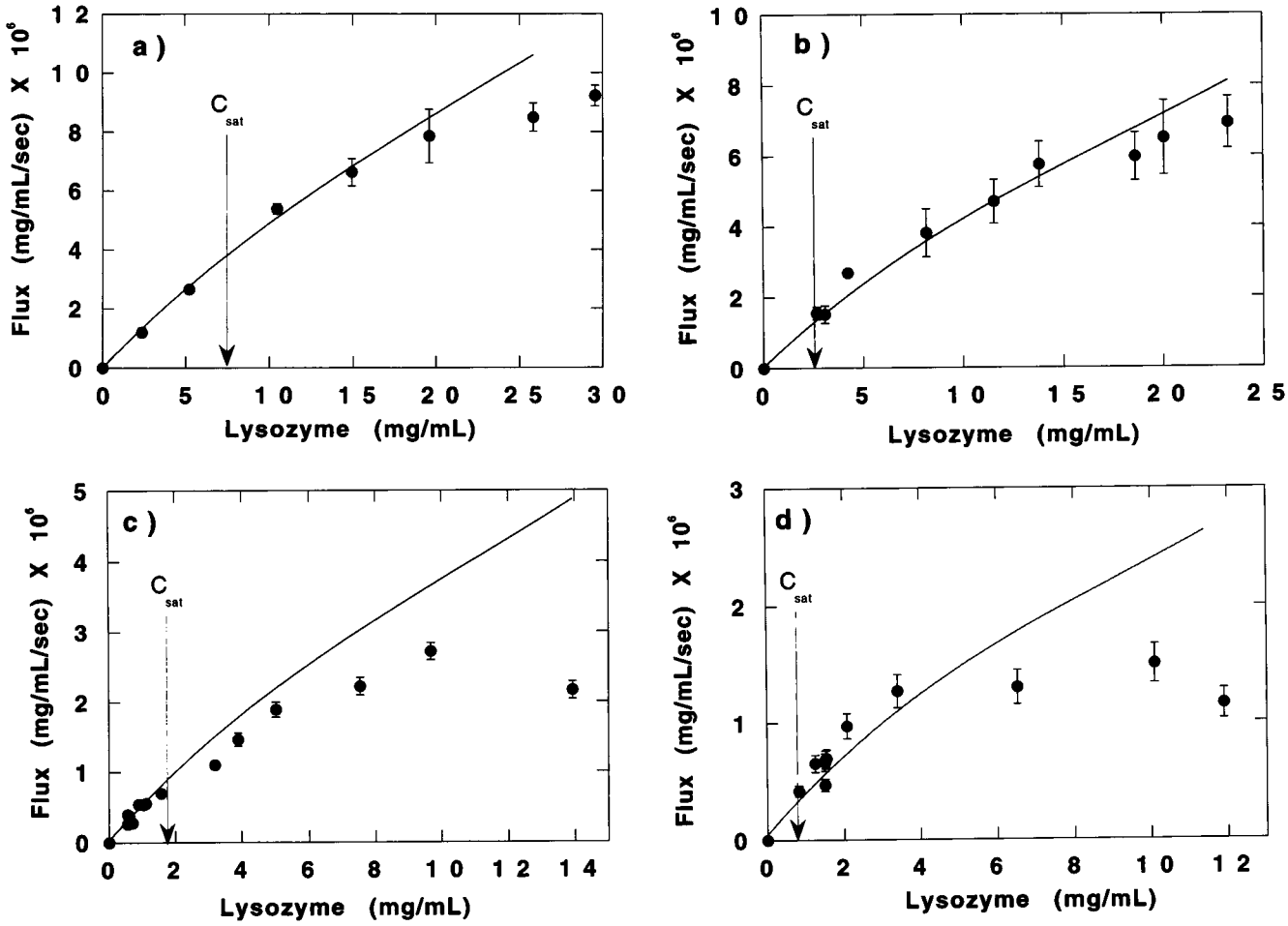

FIGURE 4 Flux measurements of lysozyme in solutions of varying NaCl concentrations. Solid lines represent calculated flux rates from Eq. 1 with monomer and dimer concentrations being derived from the dimerization constants of Table 1. The arrow represents the solubility concentration, C_{sat}, which is also shown in Table 1. *a*, 3% NaCl, pH 4.0 (0.1 M NaAc), K_1 = 295.5 M^{-1}; *b*, 4% NaCl, pH 4.0 (0.1 M NaAc), K_1 = 749.7 M^{-1}; *c*, 5% NaCl, pH 4.0 (0.1 M NaAc), K_1 = 1211 M^{-1}; and *d* 7% NaCl, pH 4.0 (0.1 M NaAc), K_1 = 7879 M^{-1}.

and

$$K_1 = \frac{[D]}{[M]^2} \quad (2)$$

where K_1 represents the dimerization constant. For each salt concentration K_1 was calculated using the entire range of concentrations as well as partial data sets using only low protein concentrations (Table 1). As seen in Fig. 4, this simple model fits the data well at low protein concentrations where one would expect monomers and dimers to be the predominant species. At high protein concentrations the calculated flux continues to increase although the measured flux levels off indicating that aggregation proceeds past the dimerization stage. Table 1 contains the values for the dimerization constants determined with both the low protein concentrations as well as those using the entire protein concentration range. Calculated fluxes using the entire data set do not fit the data well as illustrated by the higher coefficients of variation. Dimerization constants calculated using the entire range, $K_{1,all}$, are two to three times higher than those using the partial data sets. These values are overestimates of the dimerization step because they assume that all aggregate species are dimers.

DISCUSSION

The flux rates measured by our dialysis system are proportional to the concentration of permeable species. In previous work we used 15,000 MWCO membranes that were permeable to monomers only, so that the flux rate paralleled the monomer concentration. In this work the 25,000 MWCO membranes were shown to allow both monomers and dimers to transverse the membrane by using purified solutions of covalently cross-linked dimers in 1% NaCl, pH 4.0 (0.1 M NaAc). There are two factors to consider in the evaluation of the flux of the dimer across the membrane. First, the dimer was only 87% pure off of the chromatography column. Before measurements, the dimer preparations were dialyzed in a 15,000 MWCO membrane to remove the small amount of monomer present. The remaining impurities were trimers that would be too large to cross the membrane. The trimers would, however, contribute to the concentration determined by absorbance at 281.5 nm that in turn would give a higher than actual velocity constant. The second factor to be considered is the assumption that the size of a cross-linked dimer was the same as a noncovalently linked dimer. The expected molecular weight of a lysozyme dimer would be twice the monomer molecular weight of 14,400 or 28,800. However, the cross-linked dimer showed a molecular weight of 24,400 which is 18% lower than the expected molecular weight. Lower molecular weights have often been detected for cross-linked products and are thought to be due to intramolecular cross-linking that results in more rigid molecules with significantly different hydrodynamic characteristics (Marshall and Inglis, 1986). Separation procedures such as SDS-PAGE can detect this difference. For this work, we assumed that noncovalent dimers could escape from a 25,000 MWCO membrane and that their velocity constant would be the same as a covalently cross-linked dimer. All species more than or equal to a trimer of lysozyme would be retained inside the membrane.

The fact that both species can cross the membrane was built into a simple monomer to dimer equilibrium model where the measured flux was assumed to consist of monomers and dimers. This simple model only fit the data for low protein concentrations. The lack of fit of the model at higher protein concentrations indicates that higher order aggregate species are present. Addition of subsequent aggregation steps would require that we use membranes of higher MWCO to obtain aggregate concentrations of species ≥ 3.

Recently, Li et al. (1995) have developed a model for lysozyme aggregation based on the pathway of monomer <-> dimer <-> tetramer <-> octamer <-> 16-mer. This model fit measured growth rate data for lysozyme remarkably well for three sets of pH and salt concentrations. The estimated equilibrium constants from this model at pH 4.0 and 5% NaCl provided us with the corresponding calculated monomer and dimer concentrations. These concentrations were converted to f_{tot} using Eq. 1. Fig. 5 shows that the calculated flux from Li's model (*dashed line*) fits our measured flux rate much better than the simple monomer to dimer equilibrium. As expected, the addition of more steps to the aggregation pathway results in a decrease in the

TABLE 1 Calculated equilibrium constants

			Partial data set			Entire data set		
Conditions	I* mol/liter	S# mg/ml	K_1 liter/mol	CV§ %	Range¶ mg/ml	$K_{1,all}$ liter/mol	CV %	Range mg/ml
1.0% NaCl, pH 4.0, 20°C	0.271	>100				8.1	7.53	0 to 40
3.0% NaCl, pH 4.0, 20°C	0.613	7.14	295.5	15.74	0 to 14	656.5	17.03	0 to 30
4.0% NaCl, pH 4.0, 20°C	0.689	2.43	749.7	9.21	0 to 10	1085	13.43	0 to 23
5.0% NaCl, pH 4.0, 20°C	0.956	1.69	1212	18.38	0 to 5	2704	33.11	0 to 14
7.0% NaCl, pH 4.0, 20°C	1.30	0.83	7879	5.12	0 to 3	58250	35.70	0 to 11

*Ionic strength that includes the contribution due to the 0.1 M sodium acetate buffer solution.
#Solubility concentration from Cacioppo and Pusey, 1991.
§Coefficient of variation for K_1 in percentage.
¶Concentration range over which the data were modeled.

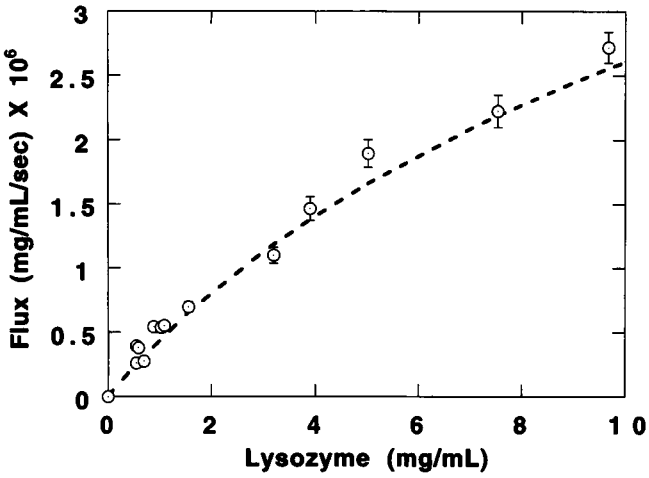

FIGURE 5 Comparison of experimental data (circo) and calculated flux (---) from the model of Li et al. (1995) for 5% NaCl, pH 4.0 (0.1 M NaAc). Li's model fits well over the entire protein concentration range whereas the simple monomer to dimer equilibrium model shown in Fig. 3 only fits only the low concentration data.

monomer concentration as larger aggregates are being produced. This decrease in the monomer concentration results in a lowering of the flux rate out of a 25,000 MWCO membrane.

Evidence that the ionic strength of the solution is a moderator of protein-protein interactions in crystallizing lysozyme solutions comes from the linear plot of the log K_1 versus $\sqrt{I}$, which is shown in Fig. 6 (R = 0.987). Lysozyme solubility decreases with increasing ionic strength that would be reflected by increasing values of K_1 as shown in the figure. The value of K_1 determined by extrapolation to zero ionic strength is 4.9×10^{-2} although the no salt constant at the ionic strength of the buffer is 0.143. These

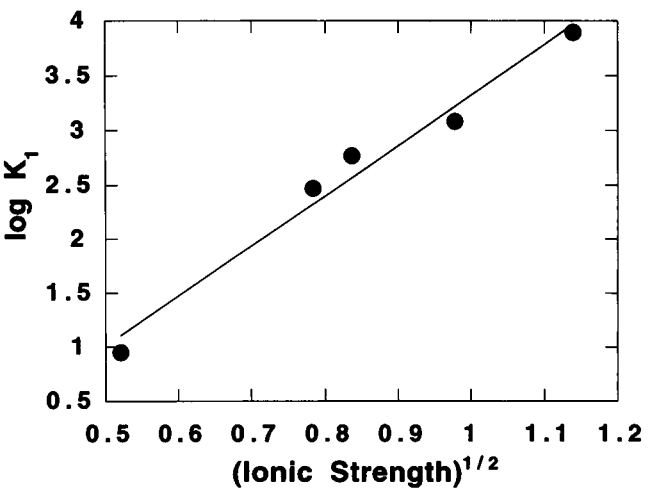

FIGURE 6 Linear plot of log K_1 versus the square root of the ionic strength (R = 0.987). Ionic strength includes the contribution of the buffer (0.1 M). The intercept gives a zero salt dimerization constant of 4.9×10^{-2} M^{-1} although an ionic strength of 0.1 gives a no salt constant of 0.143 M^{-1}.

low values for the dimerization constant illustrate the need for ionic strength moderators to induce aggregation. The essentially monodisperse nature of lysozyme at low ionic strength is reflected in the values of K_1 at low ionic strength.

Because some of the experiments were conducted in the metastable region for lysozyme, our system is not at a true equilibrium. However, assuming that reequilibration proceeds fast one can define the relevant equilibrium constants as the ratio of the product concentrations over the reactant concentrations for each step in the aggregation pathway. These expressions do not include any provision for the saturation concentration or presence or absence of a solid phase. Thus, these equilibria are equally valid in under- or oversaturated solutions and in the presence of the solid phase. Mechanisms for crystal nucleation and growth processes will be greatly influenced by the species present in solution and interpretation of face growth rates and nucleation rates must take into account aggregate distributions.

CONCLUSIONS

Dialysis kinetics measurements have been made to determine dimerization equilibrium constants for tetragonal lysozyme crystallization in medium to high salt concentrations and acidic pH. These equilibrium constants varied linearly with the square of the ionic strength of the solution. The flux was found to be due to both monomers and dimers escaping from a 25,000 MWCO membrane. The dimerization model fit the flux data well at low protein concentrations. However, at high protein concentrations there was significant deviation demonstrating the need for a more complicated aggregation model using larger aggregate species. For one set of conditions, 5% NaCl (pH 4.0), excellent agreement was found using the 1 <-> 2 <-> 4 <-> 8 <-> 16 model developed by Li et al. (1995).

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