Time Dependence of Aggregation in Crystallizing Lysozyme Solutions Probed Using NMR Self-Diffusion Measurements

William S. Price,* Fumihiko Tsuchiya,† and Yoji Arata‡

*Department of Physical Chemistry, Royal Institute of Technology, SE-100 44 Stockholm, Sweden; †Japan Food Research Laboratories, Tokyo 206-0025, Japan; and ‡Water Research Institute, Ibaraki 305-0047, Japan

ABSTRACT The time dependence of aggregation in supersaturated lysozyme solutions was studied using pulsed-gradient spin-echo NMR diffusion measurements as a function of lysozyme concentration at pH 6.0 and 298 K in the presence of 0.5 M NaCl. The measurements provide estimates of the weight-averaged diffusion coefficient of the monomeric to intermediate molecular weight lysozyme species present in the solution (very large aggregates and crystals are excluded from the average due to the NMR relaxation-weighting effects inherent in the method). The results show that the average molecular weight of the various lysozyme aggregates changed with sigmoidal kinetics and that these kinetics were strongly influenced by the initial lysozyme concentration. The visualization of the time dependence of the protein aggregation afforded by this method provides a deeper understanding of how the crystallizing conditions (especially the initial protein concentration) are related to the resulting crystals.

INTRODUCTION

The lack of methods for determining suitable conditions for protein crystallization growth is a serious impediment in protein structure determination (Kierzek et al., 1997; Riès-Kautt and Ducruix, 1997; Soumpasis and Georgalis, 1997). Lysozyme is perhaps the most studied protein with respect to crystallization. In its monomeric form it is a nearly spherical prolate ellipsoid (i.e., $a = 27.5$ Å and $b = 16.5$ Å) and has a molecular weight of 14,320 (Dubin et al., 1971). Aggregation is known to be an essential step in the crystallization process. The aggregation process and protein solubility has a complex dependence on pH, temperature, and the protein and salt concentrations. In low ionic strength solutions, lysozyme interacts mainly through a combination of electrostatic repulsion and attractive dispersion forces (Kuehner et al., 1996); however, above 0.5 M NaCl the electrostatic screening is virtually complete (Eberstein et al., 1994; Retailleau et al., 1997) and van der Waals interactions prevail.

To date, a number of methods have been used to provide information on the earliest stage of the lysozyme crystallization process (i.e., aggregation) including equilibrium ultracentrifugation (Wills et al., 1980), dialysis (Wilson et al., 1996), interferometry (Kim and Myerson, 1994; Albright et al., 1999), light and neutron scattering (Kuehner et al., 1997; Tanaka and Hayakawa, 1999; Georgalis et al., 1999), NMR relaxation dispersion studies (Raeymaekers et al., 1989), and most recently pulsed-gradient spin-echo (PGSE; also known as pulsed field gradient and DOSY) (see Price, 1996, 1997, 1998) NMR diffusion measurements (Price et al., 1999; Price, 2000).

In the present work we investigate the time dependence of aggregation in supersaturated solutions of lysozyme at pH 6 and in the presence of 0.5 M NaCl using PGSE NMR diffusion measurements. PGSE NMR provides estimates of the self-diffusion coefficient, D, which correlates with the average molecular weight of the lysozyme oligomers present.

PGSE NMR measurements of lysozyme aggregation

As we have shown previously (Price et al., 1999), in a solution containing a slowly exchanging (on the PGSE NMR time scale) unimodal distribution of low-molecular-weight lysozyme oligomeric states i (i.e., monomer, dimer, . . . i-mer) undergoing isotropic free diffusion, the echo attenuation in a PGSE NMR experiment is sensitive to the weight-averaged diffusion coefficient, viz.,

$$\ln(E) = -\gamma^2 g^2 \delta^2 (\Delta - \delta/3) \langle D \rangle_{\mathrm{W}}^{\mathrm{C}}, \qquad (1)$$

where γ is the gyromagnetic ratio, g is the magnitude and δ is the duration of the rectangular magnetic field gradient pulses, and Δ is the separation between the leading edges of the gradient pulses. Δ is the period over which diffusion is measured and it defines the time scale of the PGSE experiment. $\langle D \rangle_{\mathrm{W}}^{\mathrm{C}}$ is the weight-averaged diffusion coefficient defined by

$$\langle D \rangle_{\mathrm{W}}^{\mathrm{C}} = \frac{\sum_{\mathrm{i}} Mw_{\mathrm{i}} n_{\mathrm{i}} D_{\mathrm{i}}^{\mathrm{C}}}{\sum_{\mathrm{i}} Mw_{\mathrm{i}} n_{\mathrm{i}}}, \qquad (2)$$

where the index i is over the different lysozyme oligomers present (e.g., $i = 2$ would be dimeric lysozyme), Mw_{i} is the molar mass of the ith aggregate species and n_{i} is the number of such molecules present. We note that the diffusion coef-

Received for publication 15 August 2000 and in final form 21 November 2000.

Address reprint requests to Dr. William S. Price, Department of Physical Chemistry, Royal Institute of Technology, SE-100 44 Stockholm, Sweden. Tel.: 46-8-7908206; Fax: 46-8-7908207; E-mail: wprice@physchem.kth.se.

0006-3495/01/03/1585/06 $2.00

ficients of the lysozyme oligomers (i.e., D_i^C) inherently contain the effects of crowding (denoted by the superscript *C*) because the average spacing of the lysozyme molecules is much less than the mean-squared displacement of the particles over the time scale (i.e., Δ) of the PGSE experiment. Crowding has two consequences: first, the measured diffusion coefficient of any oligomeric species, D_i^C, will be smaller than its infinite dilution value, D_i^0, due to self-obstruction, and second, it results in some averaging of the diffusion coefficients of the different oligomers. Thus, as was noted previously (Price et al., 1999) only a single average diffusion coefficient was observed in the PGSE measurements (although if the degree of polydispersity is not high, deviations from Eq. 1 may not be very evident). Thus, simplistically assuming that we have spherical aggregates (leading to isotropic association), the theoretical apparent diffusion coefficient is given by (NB $Mw_i n_i \propto c_i i$; c_i is the molar concentration of the *i*th aggregate)

$$\langle D\rangle_W^C = \langle D\rangle_W^0 f_C(C) = \frac{\sum_i c_i i D_1^0 i^{-1/3}}{C} f_C(C) = \sum_i \alpha_i D_1^0 i^{-1/3} f_C(C), \quad (3)$$

where *C* is the total lysozyme concentration, α_i is the mole fraction of each oligomeric species and the term $f_c(C)$ accounts for the reduction in the diffusion coefficient due to crowding at a particular protein concentration (vide infra).

In developing the above model we have assumed that the exchange between different oligomeric states is slow. This assumption is reasonable because it has been found (Xavier and Willson, 1998) that antibody-lysozyme complexes have lifetimes much greater than 10^3 s and that lysozyme solutions can take very long times to reach equilibrium (Retailleau et al., 1997). But in any case, due to the ensemble averaging included in the above model, the final equation (i.e., Eq. 3) is equivalent to that for the case of fast exchange.

Only approximate models exist for accounting for the decrease in diffusion coefficient due to crowding, $f_c(C)$ (e.g., see Muramatsu and Minton, 1988; Pusey, 1991; Han and Herzfeld, 1993; Tokuyama and Oppenheim, 1994). In the present work we use the model by Tokuyama and Oppenheim (1994) to calculate $f_c(C)$. Because this model does not account for the presence of an aggregation process, it progressively overestimates the reduction in diffusion as the concentration (and degree of aggregation) increases. Furthermore, it does not account for charge effects (Pusey, 1991).

In our previous study of low-molecular-weight aggregates in lysozyme solutions (Price et al., 1999), the theory embodied in Eq. 3 provided a reasonable description of the system even though the effects of relaxation weighting were explicitly ignored in analyzing the PGSE data. In the present study, however, such effects merit more careful consideration due to the more polydisperse nature of the samples used (and correspondingly larger variation in relaxation times). The diffusion measurements in the current work were performed using the stimulated echo sequence (i.e., $\pi/2$-τ_1-$\pi/2$-τ_2-$\pi/2$-τ_1-acquire) with identical rectangular gradient pulses of duration δ and magnitude g in the first and second τ_1 periods. Thus, in addition to the attenuation due to diffusion discussed above, the signal acquired is reduced due to the effects of spin-lattice relaxation during τ_2 and spin-spin (or transverse relaxation) during the τ_1 periods. Thus, in reality, relaxation weighting should be included in Eq. 3 by including $\exp(-2\tau_1/T_{2,i})\exp(-\tau_2/T_{1,i})$, where $T_{1,i}$ and $T_{2,i}$ are the spin-lattice and spin-spin relaxation times of the NMR resonances from the *i*th resonance. As the size of an oligomer increases, so too does its overall reorientational correlation time, τ_c, and consequently both T_1 and T_2 decrease, although for very large τ_c values T_1 can start to increase. Nevertheless, the T_2 term dominates the relaxation-weighting effects, and thus the contribution to the PGSE NMR signal of an aggregate will become less than proportional to its molecular weight with increasing molecular weight and for very large oligomers/aggregates will eventually become negligible (see Price et al., 1997, for a related discussion).

The above model of relaxation weighting is really still simplistic because although the overall τ_c increases proportionally to molecular weight, the relaxation of the protein side chains, which contain most of the detected spins, decreases much more slowly due to partial averaging by the motion of the side chains themselves (e.g., see Lipari and Szabo, 1982), and thus, at least for low-molecular-weight species, the neglecting of relaxation-weighting effects is not too unreasonable. Nevertheless, as the size of an oligomer increases, its contribution to the detected NMR signal will be less than proportional to its molecular weight. Given the experimental parameters used in the present experiment, and by comparison with previous studies with immunoglobulins (unpublished results), lysozyme oligomers with $Mw \gtrsim 100{,}000$ should make only a small contribution to $\langle D\rangle_W^C$ due to the effects of relaxation weighting. We emphasize that a quantitative accounting of relaxation weighting in the lysozyme systems is very difficult, if not impossible, due to the inability to measure the relaxation times (of the resonances) of the individual oligomeric systems as the resonances of all of the oligomeric species overlap.

MATERIALS AND METHODS

Samples containing 3, 5, 6, and 7 mM lysozyme at pH 6 in aqueous (90%:10% ^1H_2O:^2H_2O) NaCl solution containing 0.5 M NaCl were prepared as previously described (Price et al., 1999). All of these solutions are supersaturated (e.g., see Fig. 4 in Riès-Kautt and Ducruix, 1997). Due to its high isoelectric point (i.e., pH 11), lysozyme has a net positive charge of 9 at the pH used in the present study (Riès-Kautt and Ducruix, 1997).

The ^{1}H PGSE NMR measurements were performed at 500 MHz using a Bruker DMX 500 spectrometer (static magnetic field = 11.7 T) as previously described (Price et al., 1999). Samples (~0.2 ml) of each sample was placed into magnetic susceptibility-matched (to water) NMR tubes (BMS-005; Shigemi, Tokyo) giving a sample height of less than 1 cm. With this sample arrangement the entire lysozyme sample remained within the radio frequency coils irrespective of whether aggregation/crystallization resulted in heterogeneous distributions (e.g., sedimentation) throughout the NMR tube during the time course of the experiment. Diffusion measurements were performed using the stimulated echo pulse sequence. The experimental parameters used were $\Delta = 34$ ms and $\delta = 5$ ms with g varied up to 0.64 T m^{-1} with $\tau_1 = 6$ ms and $\tau_2 = 27$ ms. Each diffusion measurement took ~30 min (which is fast compared with the change in diffusion coefficient with time; vide infra). Including all possible sources of error in both PGSE measurement and sample preparation the absolute accuracy of the measured diffusion coefficients is within a few percent. In each case the lysozyme sample was kept stationary in the magnet for the duration of the experiment (i.e., the PGSE measurements and the intervening periods; total, ~1 week).

RESULTS AND DISCUSSION

The change in the weight-averaged diffusion coefficient of the lysozyme solutions with time is shown in Fig. 1. An estimate of the diffusion of a lysozyme monomer at infinite dilution, $D_1^0 = 1.12 \times 10^{-10}$ m^2 s^{-1}, can be obtained by noting that

$$D_1^0 = D_1^C / f_C(C), \tag{4}$$

with f_C (1.5 mM) = 0.94 and $D_1^C \approx D_{\sim 1}^{C=1.5\ mM} = 1.05 \times 10^{-10}$ m^2 s^{-1} (see the data for 1.5 mM lysozyme at pH 6 and 298 K in Fig. 2 C in Price et al., 1999, which should be mainly monomeric lysozyme). Estimates for D_1^C for $C = 3$, 5, 6, and 7 mM were calculated from this value of D_1^0 and appropriate values for $f_C(C)$ (see Table 1) and are plotted at $t = 0$ h (open symbols) in Fig. 1. The increasingly large discrepancy between D_1^C and the initial $\langle D \rangle_W^C$ value at $t = 0$ h (i.e., $\langle D(t_0) \rangle_W^C$) with lysozyme concentration reflects increased proportions of higher oligomers. No attempt to deconvolute the effects of crowding from that of aggregation was undertaken in our analysis due to the lack of a model for crowding that includes both aggregation and charge effects as noted above. However, we note that the maximum possible effects of crowding on the diffusion for each sample is given by $f_C(C)$ at each concentration (see Table 1 and the open symbols in Fig. 1). However, as there is significant aggregation, these values are probably significant overestimates of the attenuation of the diffusion coefficient due to crowding. Hence the main effect is aggregation and not crowding.

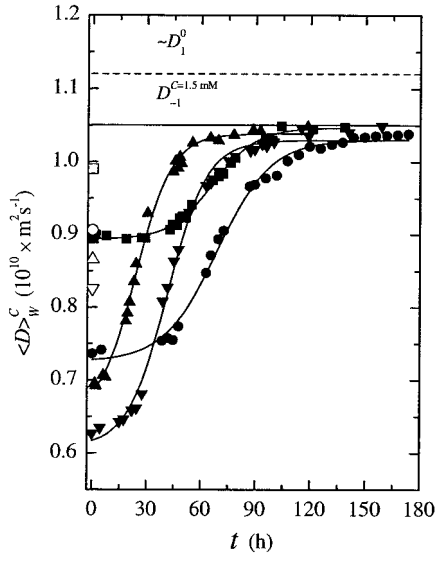

FIGURE 1 The change in the PGSE NMR-determined diffusion coefficients, $\langle D \rangle_W^C$, of the lysozyme samples (■, 3 mM; ●, 5 mM; ▲, 6 mM; ▼, 7 mM) with time at pH 6 and 298 K in 0.5 M NaCl. The open symbols at $t = 0$ h represent the corresponding D_1^C values for these concentrations. The horizontal solid line indicates $D_{\sim 1}^{C=1.5\ mM}$ and the horizontal dashed line indicates ~D_1^0 (see text). The solid lines represent the regression of Eq. 5 onto the data.

All four samples exhibited an induction period not unlike that observed in quasi-elastic light-scattering measurements of crystallizing protein solutions (Malkin and McPherson, 1993). The time dependence of each set was well described by a sigmoidal (Boltzmann) function of the form

$$\langle D \rangle_W^C = \frac{\langle D(t_0) \rangle_W^C - \langle D(t_\infty) \rangle_W^C}{1 + e^{(t - t_{sigm})/t_S}} + \langle D(t_\infty) \rangle_W^C, \tag{5}$$

where $\langle D(t_\infty) \rangle_W^C$ represents the long time value of $\langle D \rangle_W^C$, t_{sigm} is the midpoint of the sigmoidal inflection and t_S is the time scaling factor that determines the slope of the inflection. We note that the observed sigmoidal kinetics may imply an autocatalytic mechanism (Pasternack et al., 1998). The inflection period corresponds to a coarsening or Ostwald ripening stage. Eq. 5 was regressed onto each of the data sets; the results are shown in Fig. 1 and the corresponding parameter estimates obtained are tabulated in Table 1. The induction period was quite long for the 3 and 5 mM samples but was very much shorter for the 6 and 7 mM samples. Also the slope of the inflection was very much greater for the two most concentrated samples (see Table 1). At long times the measured diffusion coefficient of each of the four samples approached that of a 1.5 mM lysozyme solution ($D_{\sim 1}^{C=1.5\ mM}$). After removing the samples from the spectrometer, visual observation revealed that the 3 and 5 mM samples produced a small number of large crystals whereas the 6 and 7 mM samples produced many small crystals (data not shown). In all samples the crystals were columnar with the long axis oriented along the static magnetic field in agreement with previous reports (e.g., see Wakayama, 1998).

The changes in one-dimensional spectra (acquired directly after a $\pi/2$ pulse) of the 7 mM lysozyme sample with time after sample preparation are shown in Fig. 2. Initially the sample contains a polydisperse mixture of low- to medium-weight lysozyme oligomers (i.e., of the order of tet-

TABLE 1 Decrease in diffusion coefficient for the four samples

Sample	$f(C)$	$\langle D(t_0)\rangle_W^C$ ($\times 10^{-10}$ m^2 s^{-1})	$\langle D(t_\infty)\rangle_W^C$ ($\times 10^{-10}$ m^2 s^{-1})	t_{sigm} (h)	t_S (h)	m_{sigm} ($\times 10^{-17}$ m^2 s^{-2})
3 mM	0.884	0.89 ± 0.00	1.05 ± 0.00	66.5 ± 0.8	11.8 ± 0.7	9.4
5 mM	0.809	0.72 ± 0.01	1.03 ± 0.00	68.9 ± 1.2	14.6 ± 1.0	14.8
6 mM	0.773	0.65 ± 0.01	1.04 ± 0.00	23.9 ± 0.9	9.9 ± 0.8	27.4
7 mM	0.737	0.61 ± 0.01	1.03 ± 0.00	41.4 ± 0.9	10.8 ± 0.8	27.0

The decrease in diffusion coefficient, $f(C)$, for the four samples determined from the model of Tokuyama and Oppenheim (1994) and taking the partial specific volume of lysozyme as 0.75 ml/g and the results of regressing the sigmoidal function (Eq. 5) onto the data presented in Fig. 1. The slope, m_{sigm}, at the mid point of the inflection (i.e., $m_{sigm} = (\langle D(t_\infty)\rangle_W^C - \langle D(t_0)\rangle_W^C)/(4t_S)$) is also shown.

ramer or greater but not very large aggregates or crystals) and there is very little fine structure in the spectrum, which is consistent with the long τ_c values expected for oligomers of this size. However, with time some of the lysozyme proceeds to larger, more slowly reorienting, oligomeric states and ultimately leaves the solution state thereby forming a two-state system (i.e., large aggregates/crystals in a solution of lower-molecular-weight aggregates). The large aggregates/crystals can be classed as solids in the NMR sense due to their very long τ_c and are consequently NMR invisible. As the lysozyme aggregates out of solution to form solid lysozyme, the concentration of the lysozyme in solution is decreased (note that the total lysozyme concentration in the sample is, of course, unchanged). This can be clearly seen by noting the decrease in the size of the lysozyme signal with time (e.g., compare the $t = 18$ to the $t = 88$ h spectra). The smaller lysozyme aggregates in solution are, however, NMR visible and thus there is more fine structure evident in the $t = 88$ h spectrum.

The decrease in total lysozyme signal would be even more pronounced if the spectra had been acquired with the stimulated echo sequence due to the inherently greater relaxation weighting as discussed above. Hence, as the aggregation process proceeds the NMR signal obtained with the stimulated echo sequence arises predominantly from the monomers/small oligomers. Or, in other words, the stimulated echo acts as a molecular weight filter rejecting the resonances from the high-molecular-weight species (i.e., macro aggregates and crystals) on the basis of their shorter relaxation times. The lower concentration also means that the effects of crowding are diminished and the measured diffusion coefficient increases as observed in Fig. 1.

Following the Stokes-Einstein equation it can be shown that the infinite dilution diffusion coefficient of the oligomers decreases according to the inverse cube root of their molecular weight (i.e., $D_i \propto Mw_i^{-1/3}$). Thus it is possible to recast the diffusion data of Fig. 1 in terms of the average molecular weight, $\langle Mw\rangle^C$ (see Fig. 3). Presented in this form the interpretation of the longer induction periods and smaller maximum slopes during the inflection observed for the 3 and 5 mM samples becomes more straightforward. Both of these effects likely result from there being comparatively few aggregates formed that exceeded some critical

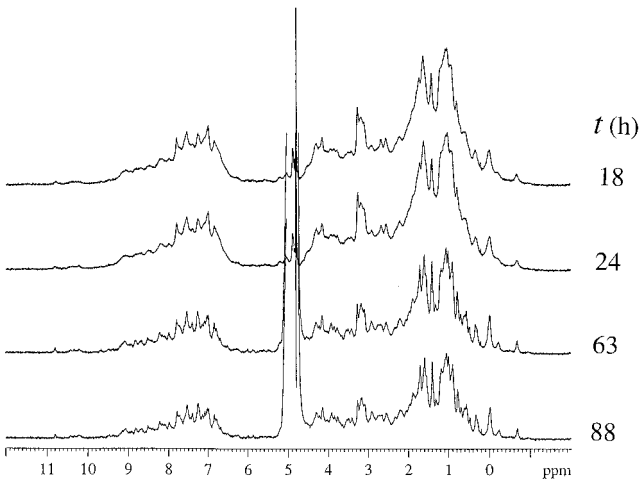

FIGURE 2 Change in the ^{1}H NMR spectra (i.e., $\pi/2$ − acquire) of the 7 mM lysozyme sample at pH 6 in 0.5 NaCl acquired at 298 K with time after sample preparation. All of the spectra were acquired under the same conditions and are presented with the same vertical scale.

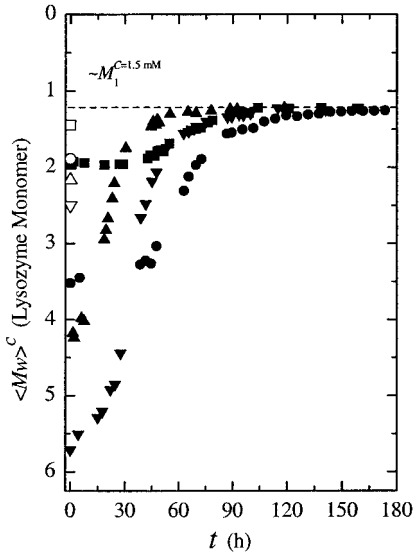

FIGURE 3 The lysozyme diffusion data replotted in terms of the average molecular weight, $\langle Mw\rangle^C$. The symbols have the same meaning as in Fig. 1.

value (i.e., to be nuclei) and it also explains why ultimately these samples produce a small number of large crystals. Conversely, the short induction periods and steeply sloped inflections of the 6 and 7 mM samples indicate that many aggregates exceeded the critical value and consequently these samples produced many small crystals. This is not surprising as the chance of lysozyme monomers encountering each other via a diffusive process increases rapidly with the concentration. Thus, the present data are consistent with previous reports where it has been suggested that the critical nucleus most likely consists of four monomers and that the growth unit is likely to be the octamer (Bessho et al., 1994; Kierzek et al., 1997; Boué et al., 1993; Nadarajah et al., 1995, 1997).

Concluding remarks

The estimate of $\langle D \rangle_W^C$ provided by PGSE NMR measurements gives a wealth of information about the solution behavior (i.e., average aggregate size and diffusion coefficient) of lysozyme during the early stages of crystallization. Importantly, PGSE gives estimates of the self-diffusion coefficient, which is easier to interpret than the mutual diffusion coefficient that is obtained in light-scattering experiments. Specifically the time course of $\langle D \rangle_W^C$ allows a deeper understanding between the initial crystallization conditions and the crystals that ultimately result. In the present preliminary study, each sample was kept in the magnet for the duration of the experiment (~1 week). Because the magnetic field may induce orientation of the lysozyme molecules thereby stimulating nonisotropic aggregation and because it has been reported that magnetic fields can cause less nucleation and slower lysozyme crystal growth rates (Sazaki et al., 1997; Yanagiya et al., 2000), it would be interesting to investigate the change of $\langle D \rangle_W^C$ if the sample were removed from the magnet between PGSE measurements and/or if the measurements were conducted at different magnetic field strengths.

W.S.P. thanks the Swedish Council for International Cooperation in Research and Higher Education (STINT) for financial support. Dr. István Furó is thanked for useful discussions.

REFERENCES

Albright, J. G., O. Annunziata, D. G. Miller, L. Paduano, and A. J. Pearlstein. 1999. Precision measurements of binary and multicomponent diffusion coefficients in protein solutions relevant to crystal growth: lysozyme chloride in water and aqueous NaCl at ph 4.5 and 25°C. *J. Am. Chem. Soc.* 121:3256–3266.

Bessho, Y., M. Ataka, M. Asai, and T. Katsura. 1994. Analysis of the crystallization kinetics of lysozyme using a model with polynuclear growth mechanism. *Biophys. J.* 66:310–313.

Boué, F., F. Lefaucheux, M. C. Robert, and I. Rosenman. 1993. Small angle neutron scattering study of lysozyme solutions. *J. Cryst. Growth.* 133:246–254.

Dubin, S. B., N. A. Clark, and G. B. Benedek. 1971. Measurement of the rotational diffusion coefficient of lysozyme by depolarized light scattering: configuration of lysozyme in solution. *J. Chem. Phys.* 54: 5158–5164.

Eberstein, W., Y. Georgalis, and W. Saenger. 1994. Molecular interactions in crystallizing lysozyme solutions studied by photon correlation spectroscopy. *J. Cryst. Growth.* 143:71–78.

Georgalis, Y., P. Umbach, W. Saenger, B. Ihmels, and D. M. Soumpasis. 1999. Ordering of fractal clusters in crystallizing lysozyme solutions. *J. Am. Chem. Soc.* 121:1627–1635.

Han, J., and J. Herzfeld. 1993. Macromolecular diffusion in crowded solutions. *Biophys. J.* 65:1155–1161.

Kierzek, A. M., W. M. Wolf, and P. Zielenkiewicz. 1997. Simulations of nucleation and early growth stages of protein crystals. *Biophys. J.* 73:571–580.

Kim, Y.-C., and A. S. Myerson. 1994. Diffusivity of lysozyme in undersaturated, saturated and supersaturated solutions. *J. Cryst. Growth.* 143: 79–85.

Kuehner, D. E., H. W. Blanch, and J. M. Prausnitz. 1996. Salt-induced protein precipitation: phase equilibria from an equation of state. *Fluid Phase Equilibria.* 116:140–147.

Kuehner, D. E., C. Heyer, C. Rämsch, U. M. Fornefeld, H. W. Blanch, and J. M. Prausnitz. 1997. Interactions of lysozyme in concentrated electrolyte solutions from dynamics light-scattering measurements. *Biophys. J.* 73:3211–3224.

Lipari, G., and A. Szabo. 1982. Model-free approach to the interpretation of nuclear magnetic resonance relaxation in macromolecules. I. Theory and range of validity. *J. Am. Chem. Soc.* 104:4546–4559.

Malkin, A. J., and A. McPherson. 1993. Light scattering investigations of protein and virus crystal growth: ferritin, apoferritin and tobacco mosaic virus. *J. Cryst. Growth.* 128:1232.

Muramatsu, N., and A. P. Minton. 1988. Tracer diffusion of globular proteins in concentrated protein solutions. *Proc. Natl. Acad. Sci. USA.* 85:2984–2988.

Nadarajah, A., E. L. Forsythe, and M. L. Pusey. 1995. The averaged face growth rates of lysozyme crystals: the effect of temperature. *J. Cryst. Growth.* 151:163–172.

Nadarajah, A., M. Li, and M. L. Pusey. 1997. Growth mechanism of the (110) face of tetragonal lysozyme crystals. *Acta Cryst.* D53:524–534.

Pasternack, R. F., E. J. Gibbs, P. J. Collings, J. C. dePaula, L. C. Turzo, and A. Terracina. 1998. A nonconventional approach to supramolecular formation dynamics. the kinetics of assembly of DNA-bound porphyrins. *J. Am. Chem. Soc.* 120:5873–5878.

Price, W. S. 1996. Gradient NMR. *In* Annual Reports on NMR Spectroscopy. G. A. Webb, editor. Academic Press, London. 51–142.

Price, W. S. 1997. Pulsed field gradient NMR as a tool for studying translational diffusion. I. Basic theory. *Concepts Magn. Reson.* 9:299–336.

Price, W. S. 1998. Pulsed field gradient NMR as a tool for studying translational diffusion. II. Experimental aspects. *Concepts Magn. Reson.* 10:197–237.

Price, W. S. 2000. NMR gradient methods in the study of proteins. *In* Annual Reports on the Progress in Chemistry, Part C. G. A. Webb, editor. Royal Society of Chemistry, London. 3–53.

Price, W. S., H. Ide, M. Ishikawa, and Y. Arata. 1997. Intensity changes in ^{1}H-NMR micro-images of plant materials exposed to subfreezing temperatures. *Bioimages.* 5:91–99.

Price, W. S., F. Tsuchiya, and Y. Arata. 1999. Lysozyme aggregation and solution properties studied using PGSE NMR diffusion measurements. *J. Am. Chem. Soc.* 121:11503–11512.

Pusey, P. N. 1991. Colloidal suspensions. *In* Liquids, Freezing and Glass Transition. Elsevier, Amsterdam. 763–941.

Raeymaekers, H. H., H. Eisendrath, A. Verbeken, Y. van Haverbeke, and R. N. Muller. 1989. Nuclear magnetic relaxation dispersion in protein solutions as probe for protein transformation: example, the dimerization of lysozyme. *J. Magn. Reson.* 85:421–425.

Retailleau, P., M. Riès-Kautt, and A. Ducruix. 1997. No salting-in of lysozyme chloride observed at low ionic strength over a large range of pH. *Biophys. J.* 73:2156–2163.

Riès-Kautt, M., and A. Ducruix. 1997. Inferences drawn from physicochemical studies of crystallogenesis and precrystalline state. *Methods Enzymol.* 276:23–59.

Sazaki, G., E. Yoshida, H. Komatsu, T. Nakada, S. Miyashita, and K. Watanabe. 1997. Effects of a magnetic field on the nucleation and growth of protein crystals. *J. Cryst. Growth.* 173:231–234.

Soumpasis, D. M., and Y. Georgalis. 1997. Potential of mean force treatment of salt-mediated protein crystallization. *Biophys. J.* 72: 2770–2774.

Tanaka, S., and R. Hayakawa. 1999. Size and number density of precrystalline aggregates in lysozyme crystallization process. *J. Chem. Phys.* 111:10330–10337.

Tokuyama, M., and I. Oppenheim. 1994. Dynamics of hard-sphere suspensions. *Phys. Rev. E.* 50:R16–R19.

Wakayama, N. I. 1998. Quantitative study of crystallization kinetics of hen egg-white lysozyme using magnetic orientation. *J. Cryst. Growth.* 191: 199–205.

Wills, P. R., L. W. Nichol, and R. J. Siezen. 1980. The indefinite self-association of lysozyme: consideration of composition-dependent activity coefficients. *Biophys. Chem.* 11:71–82.

Wilson, L. J., L. Adcock-Downey, and M. L. Pusey. 1996. Monomer concentrations and dimerization constants in crystallizing lysozyme solutions by dialysis kinetics. *Biophys. J.* 71:2123–2129.

Xavier, K. A., and R. C. Willson. 1998. Association and dissociation kinetics of anti-hen egg white lysozyme monoclonal antibodies hyhel-5 and hyhel-10. *Biophys. J.* 74:2036–2045.

Yanagiya, S., G. Sazaki, S. D. Durbin, S. Miyashita, K. Nakajima, H. Komatsu, K. Watanabe, and M. Motokawa. 2000. Effects of a magnetic field on the growth rate of tetragonal lysozyme crystals. *J. Cryst. Growth.* 208:645–650.