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Salt-Induced Aggregation of Lysozyme Studied by Cross-Linking with Glutaraldehyde: Implications for Crystal Growth

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Abstract

Glutaraldehyde cross-linking followed by sodium dodecyl sulfate polyacrylamide gel electrophoresis has been used to detect aggregates of lysozyme in solutions which lead to crystals. In solutions of varying NaCl content, the number of aggregates was found to be related to the ionic strength of the solution. Solutions of 1% NaCl, pH 4.0 were monomeric while those containing 7-15% NaCl, pH 4.0 were shown to be as much as 36% aggregated and 64% monomeric. The aggregates detected at the highest salt and protein concentration studied were composed of dimers, trimers and tetramers. The aggregates increased by addition of single units suggesting the aggregation pathway to be that of monomer addition. The kinetics of the cross-linking reaction were slow preventing a study of either the time dependence of aggregation or the effect of temperature on aggregate distributions. Comparison of the total aggregate concentrations for NaCl and Na_2SO_4 showed that the concentration of aggregates was related to the ionic strength of the solution suggesting that in both crystallization and precipitation, electrostatic shielding of like-charged protein molecules is necessary in order for aggregation to occur.

1. Introduction

Many proteins are crystallized from inorganic salts which facilitate crystallization through removal of electrostatic barriers between protein molecules. From studies on the growth rate of protein crystals under different conditions, it is known that protein crystal growth requires supersaturation ratios significantly larger than those encountered with small-molecule crystal growth (Forsythe & Pusey, 1994). One possible explanation of these large supersaturation ratios is that aggregation is a necessity for nuclei formation or that lysozyme crystals grow by addition of pre-formed aggregates. However, it has not been shown conclusively that these aggregates are present in crystallizing solutions.

Currently in the field of protein crystal growth there is experimental evidence both supporting and disputing the presence of structured aggregates in crystalline solutions. For lysozyme, evidence supporting the aggregation theory comes from dialysis kinetics (Wilson, Adcock & Pusey, 1993), neutron scattering (Boue, Lefaucheux, Robert & Rosenman, 1993), sedimentation equilibrium (Sophianopoulos & Van Holde, 1961, 1964), nuclear magnetic resonance (Shindo, Cohen & Rupley, 1977), light scattering intensity (Pusey, 1991) and calorimetry (Banerjee, Pogolotti & Rupley, 1975). Some of these studies were carried out prior to 1980 when the conditions used for studying lysozyme aggregation were close to physiological ionic strength and neutral pH. More recently, investigators have studied acidic pH and high ionic strength since these conditions lead to tetragonal crystal growth. Many of the early studies showed clearly that lysozyme aggregates in undersaturated solutions.

Evidence against aggregation primarily comes from studies using quasi-elastic light scattering (QELS) (Veesler, Marcq, Lafont, Astier & Boistelle, 1994; Muschol, 1994). Using QELS, two groups have shown that lysozyme aggregates in the early stages of crystal growth but the solutions are monomeric during the growth phase (Azuma, Tsukamoto & Sunagawa, 1989; Mikol, Hirsch & Giegé, 1989). However, QELS is severely limited by its inability to make direct measurements of the concentration of discrete aggregate species present in a polydisperse solution. Furthermore, QELS experiments, costly in both equipment and time, are further impaired by their requirement of clean solutions for accurate measurements. This is an impediment for protein crystal growth experiments, often requiring supersaturated solutions, by their very nature unstable and subject to particulate (*i.e.* crystalline) contamination.

We report in this paper the adaptation of chemical cross-linking with glutaraldehyde to study the aggregation of lysozyme in supersaturated solutions. This method covalently links proteins in close proximity to each other and allows separation of the aggregate species. Because of the weak attraction between protein molecules any attempt to separate the aggregates for detection without this covalent link would result in

dissociation of the aggregates into monomers to meet the equilibrium criteria. We have studied the aggregation of lysozyme in NaCl and Na_2SO_4 at acidic pH. Our results clearly indicate that supersaturated solutions of lysozyme are aggregated and that the aggregate distributions are dependent upon the salt content of the solution.

2. Materials and methods

2.1. *Materials*

Chicken egg-white lysozyme containing approximately 95% protein and *Micrococcus lysodeikticus* were purchased from Sigma Chemical Co. Buffer components, salts, glutaraldehyde [50%(*w*/*w*)] and hydrazine [95%(*w*/*w*)] were all reagent grade from Fisher Scientific. Spectra/Por cellulose ester sterile DispoDialyzer bags of 6000–8000 and 15 000 molecular-weight cutoffs (MWCO's) were from Spectrum Medical Industries, Inc. Electrophoresis reagents were from Pharmacia. Precast gels (PhastGel Gradient 8–25) with six to eight lanes were purchased from Pharmacia along with the sodium dodecyl sulfate buffer strips. Pharmacia's electrophoresis low-molecular-weight calibration kit was used for protein standards. Coomassie Blue development of the gels employed PhastGel Blue R tablets from Pharmacia.

2.2. *Preparation of lysozyme solutions*

Lysozyme from chicken egg white (M_r = 14 400 Da) was purified using the double-dialysis technique (Wilson & Suddath, 1992). Approximately 1.0 g of lysozyme was dissolved in 10 ml of distilled water and the lysozyme solution was sealed inside a 6000–8000 MWCO dialysis bag. This lysozyme solution was transferred into a 15 000 MWCO dialysis tubing and dialyzed against 350 ml distilled water. The outer solution was collected after 5–7 d and concentrated by ultrafiltration. The protein concentration was determined spectrophotometrically at 281.5 nm, using an absorption coefficient ($A^{1\%}$ 1 cm) of 26.4 (Aune & Tanford, 1969). The purified enzyme was checked for purity by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) and found to be 99% pure.

2.3. *Cross-linking reaction*

The protein was cross-linked with glutaraldehyde according to Hermann, Rudolph & Jaenicke (1979), with some modifications. All cross-linking reactions were conducted at pH 4.0 in 0.1 *M* NaAc buffer and uncontrolled room temperature of approximately 295 K. All salt solutions were made by dissolving salts into 0.1 *M* NaAc buffer pH 4.0 and adjusting the pH back to 4.0. Purified lysozyme was diluted with 0.1 *M* NaAc buffer and buffered 20% NaCl solutions so that the required concentrations of NaCl and lysozyme were obtained. After incubation with the salt solution for timed intervals of 10–60 min, the solutions were reacted with 50%(*w*/*w*) glutaraldehyde in capped centrifuge tubes. The total volume of the reaction solution was 1000 ml. The concentrations of lysozyme used in this study were 0–3 mg ml^{-1} and the concentration of glutaraldehyde applied was always a 1000-fold molar excess over the lysozyme concentrations. The reactions were stopped after 10 min by adding a tenfold molar excess of hydrazine to glutaraldehyde which depleted any unreacted glutaraldehyde. After reaction, the reaction products were dialyzed against distilled water overnight to remove the salts, buffers and additional reaction products using 6000–8000 MWCO membranes.

2.4. *Enzyme assay*

Covalently cross-linked dimers of lysozyme were prepared by addition of 200 µl of 70% glutaraldehyde to 5 ml of 20 mg ml^{-1} lysozyme in 3% NaCl (0.1 *M* NaAc, pH 4.0). This preparation was allowed to react for 5 min before being loaded onto a 120 × 2.5 cm size-exclusion column of G-50 Sephadex and eluted at 0.2 ml min^{-1} with 3% NaCl (0.1 *M* NaAc, pH 4.0). The fractions containing peaks I, II and III were pooled and analyzed with SDS–PAGE (gel not shown). Fractions corresponding to peak III were identified as dimers by their molecular weight from SDS–PAGE (gel not shown). From the same gel the purity of the dimer was found to be 94%. These purified dimers were analyzed for enzyme activity following the procedure of Davies, Neuberger & Wilson (1969). Exactly 30 µl of 1 mg ml^{-1} protein (0.1 *M* phosphate, pH 7.0) was mixed with 0.90 ml of 0.30 mg ml^{-1} *Micrococcus lysodeikticus* at 293 K. Spectrophotometric analysis of this mixture at 450 nm showed a decrease in A_{450} with time. The enzymatic activity was calculated by dividing the slope (ΔA_{450} min^{-1}) between 1.0 and 4.5 min by the number of milligrams of protein and multiplying by the dilution factor.

2.5. *Separation and quantitation*

SDS–PAGE was performed with the PhastSystem according to the manufacturer's recommendations using PhastGel 8–25% gradient gels and PhastGel SDS buffer strips. After electrophoresis, the gels were stained with Coomassie Brilliant Blue R-250. The gels were scanned into Millipore's Bioimage Densitometry system running on a Sun workstation. The integrated intensity of the bands was found to be proportional to protein concentration over the 0–3 mg ml^{-1} concentration range with an R^2 = 0.994 showing that the Coomassie Blue dye bound to the protein in a manner proportional to the concentration. Pharmacia's electrophoresis low-

Table 1. *Activity assays on purified monomers and dimers*

	Trial	Slope (ΔA_{450} min^{-1})	R^2	Activity (units mg^{-1})
Monomer	1	−0.147	0.947	57336.9
	2	−0.140	0.949	54602.2
	3	−0.139	0.946	54212.6
		Mean −0.142 ± (2.8%)		Mean 55884 ± (3.1%)
Dimer	1	−0.127	0.951	32095.0
	2	−0.124	0.954	31336.9
	3	−0.132	0.952	33358.6
		Mean −0.128 ± (3.2%)		Mean 32264 ± (3.2%)

molecular-weight calibration kit was used for molecular-weight determination of the aggregates.

3. Results

3.1. *Demonstration of enzymatically active cross-linked dimers*

Table 1 shows the results of the turbidity assay for lysozyme activity. Fresh solutions of monomeric lysozyme gave an average activity rating of 55 384 units (mg protein)$^{-1}$. This is 2% lower than the manufacturer's rating of 56 500 units (mg protein)$^{-1}$ and suggests the assay was performed correctly. Next, purified covalent dimers were assayed following the same procedure. The average activity of the dimers was found to be 32 264 units (mg protein)$^{-1}$. This represents a decrease in activity of 42%.

3.2. *Detection of aggregates*

The conditions used to cross-link the lysozyme solutions were chosen while considering two factors. First, high protein concentrations and high glutaraldehyde concentrations can result in non-specific inter-aggregate cross-linking and erroneously high aggregate concentrations. For this reason, the molar ratio of glutaraldehyde to protein concentration was kept constant and the total protein concentration was kept low. Second, low ionic strength acidic solutions of lysozyme have been shown to be monomeric by light scattering (Bishop, Federicks, Howard & Sawada, 1992) and dialysis kinetics (Wilson & Pusey, 1992). Therefore, if the reaction time and glutaraldehyde to protein molar ratio chosen do not produce aggregates over the protein concentration range studied then no inter-aggregate cross-linking is occurring. We found a molar ratio of 1000:1 moles of glutaraldehyde to moles of protein at a reaction time of 10 min yielded no aggregates in lysozyme solutions containing 1% NaCl. The reaction was then carried out at 7, 10 and 15% NaCl. Aggregates were observed on SDS–PAGE gels beginning at 2 mg ml^{-1} and 7% NaCl and are shown in

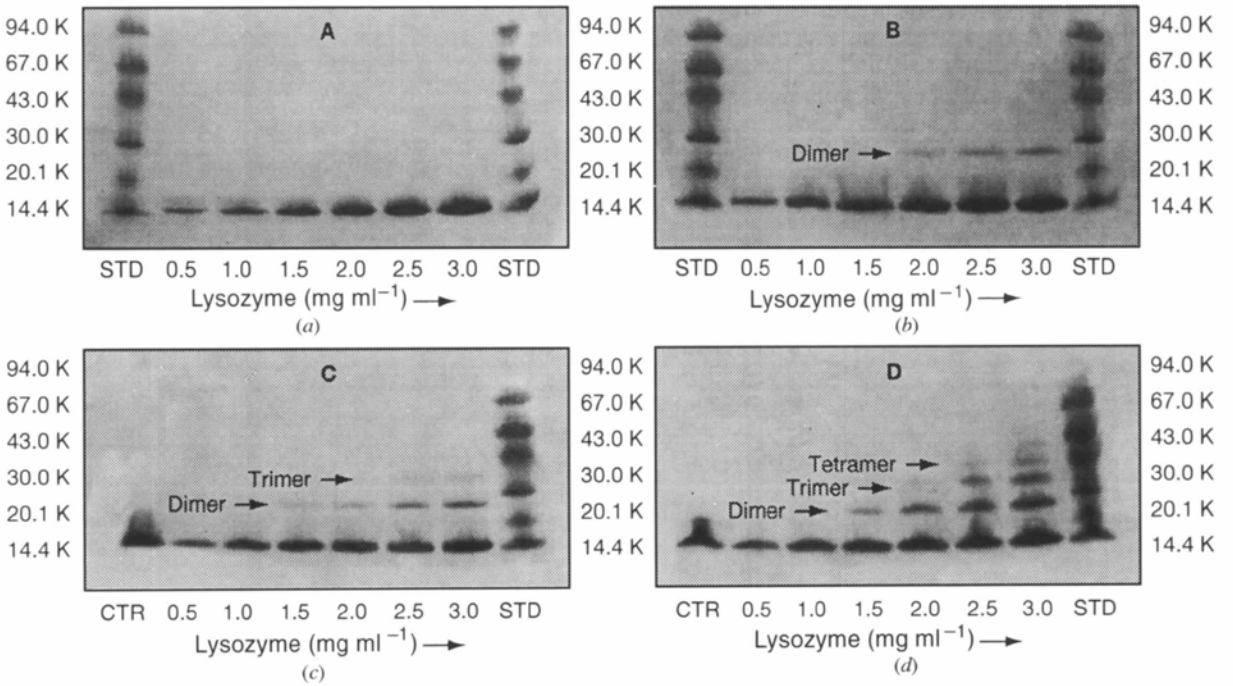


Fig. 1. SDS–PAGE of cross-linked lysozyme in 0.1 *M* NaAc, pH 4.0 buffer and (*a*) 1% NaCl, (*b*) 7% NaCl, (*c*) 10% NaCl and (*d*) 15% NaCl. Cross-linking was carried out at lysozyme concentrations ranging from 0 to 3 mg ml^{-1} at 295 K with a 10 min incubation time and a 1000:1 glutaraldehyde to protein molar ratio. Deactivation of the glutaraldehyde was initiated after the incubation period by addition of hydrazine to make a 10:1 molar ratio of hydrazine to glutaraldehyde. All standards (STD) were Pharmacia's low-molecular-weight calibration kit. Control lanes (CTR) were lysozyme without glutaraldehyde to demonstrate purity.

Fig. 1. At the higher salt concentrations, aggregates were detected at even lower protein concentrations. The total percentage of aggregates was represented on a graph as a function of protein concentration and is shown in Fig. 2. The percentage of aggregates increases with both lysozyme and the salt concentration of the solution. For the highest salt concentration studied, quantitation of the bands on the gels revealed that the solutions were as much as 36% aggregated and 64% monomeric. Also the aggregates increased in increments of single molecules, *i.e.*, dimer → trimer → tetramer. The molecular weights of the aggregates were found to be 24.4, 34.9 and 43 K for the dimer, trimer and tetramer, respectively. This is low when compared with the ideal multiple molecular weight of the monomer, 28.8, 43.2 and 57.6 K for the dimer, trimer and tetramer, respectively. The molecular weights of the cross-linked products depend on the arrangements of intermonomer cross-links. For example, a compact dimer can be formed if three cross-links are made between protein molecules instead of only one. There are more structural possibilities for a trimer and tetramer leading to different apparent molecular weights. It is significant to note that only one structural type of each oligomer was detected. The molecular weight of the monomer in the cross-linked batch was found to be 14.2 K which is slightly lower than the 14.4 K found in glutaraldehyde-free solutions. The lower molecular weight monomer suggests a small degree of intramolecular weight cross-linking which may exert a physical constraint on a denatured SDS-monomer complex thereby reducing the hydrodynamic volume and giving a smaller apparent molecular weight by SDS-PAGE.

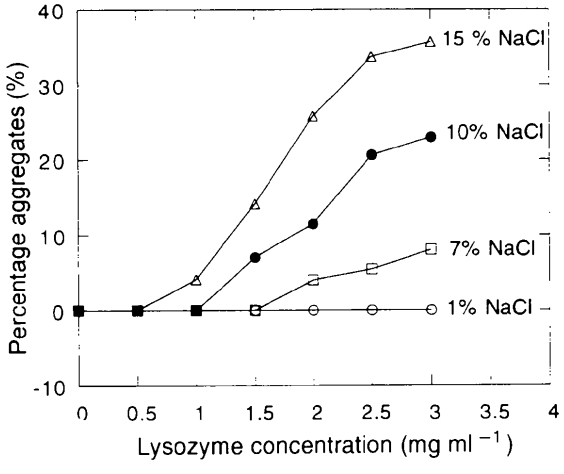
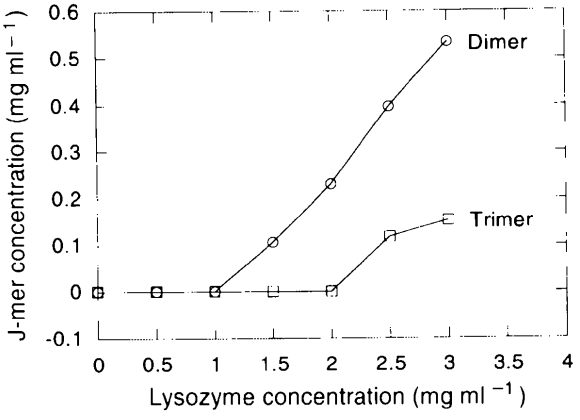


Fig. 2. Relative amounts of total aggregates determined from densitometry of gels shown in Fig. 1. Percent of total aggregates determined by summation of the percentage of total integrated intensity of individual aggregate bands using Millipore's BioImage densitometry system.

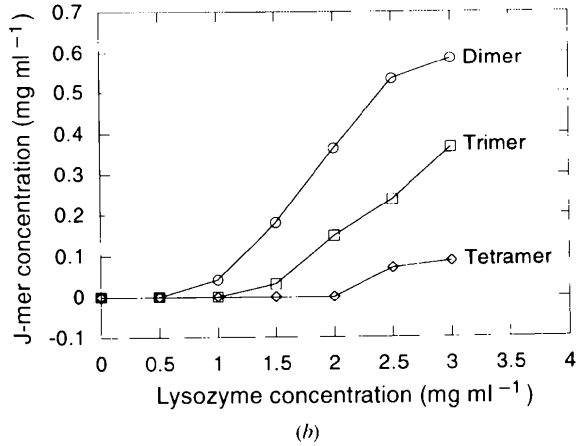
The aggregate distribution for the 10 and 15% NaCl solutions are shown in Figs. 3(*a*) and 3(*b*), respectively. For the 10% NaCl solutions, we found only two aggregate species present, specifically, dimers and trimers. The dimers did not appear until after a total lysozyme concentration of 1 mg ml^{-1} while the trimers appeared after 2 mg ml^{-1}. In 15% NaCl, we detected three aggregate species: dimers, trimers and tetramers. The same profile is seen for the 15% NaCl solutions as the 10% NaCl solutions except that the appearance of dimers and trimers occurs at lower protein concentration.

3.3. *Kinetics of cross-linking and aggregation reactions*

The time between the introduction of NaCl into the protein solution and fixing with glutaraldehyde was varied from 10 to 60 min under identical solution conditions (3.0 mg ml^{-1}, 10% NaCl, pH 4.0). The



(*a*)



(*b*)

Fig. 3. Aggregate distributions for (*a*) 10% NaCl and (*b*) 15% NaCl. Concentrations were determined by multiplying the total protein concentration by the fraction of aggregate obtained from densitometry of the gels in Fig. 1.

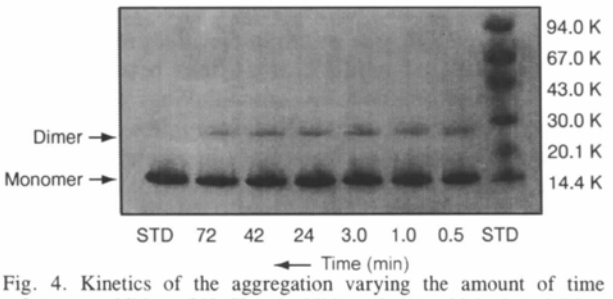
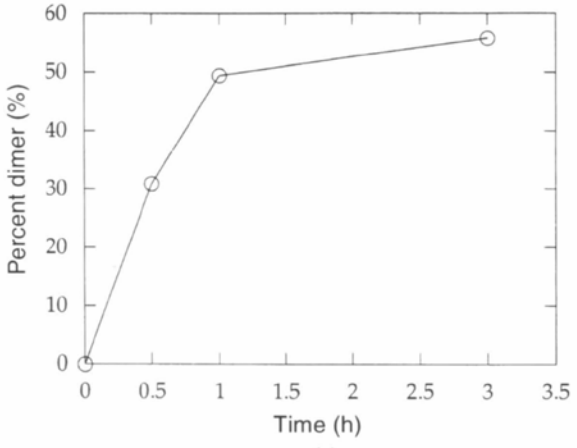


Fig. 4. Kinetics of the aggregation varying the amount of time between addition of NaCl and addition of glutaraldehyde to initiate cross-linking. Lysozyme concentration was 3.0 mg ml^{-1} with concentrated buffered NaCl added to make a 10% NaCl solution. Each lane shows a constant number of dimers (15 ± 2%) indicating that the aggregation reaction is faster than the cross-linking reaction. Control lane (CTR) was lysozyme without glutaraldehyde to demonstrate purity.



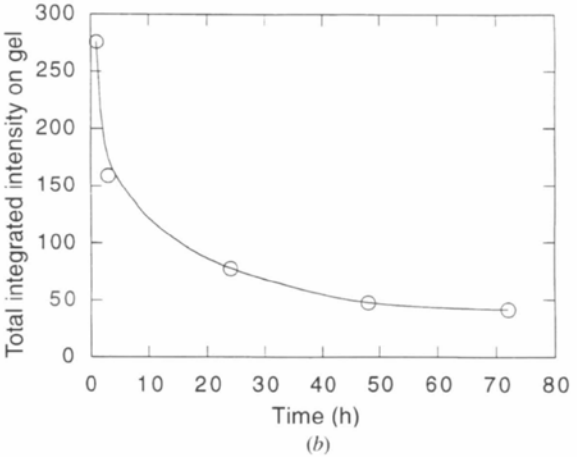


Fig. 5. Kinetics of cross-linking reaction studied by varying the incubation time of glutaraldehyde. Lysozyme concentration was 1.5 mg ml^{-1}. Buffered 20% NaCl was added to make a 7% NaCl solution followed by immediate addition of glutaraldehyde. (*a*) Percent dimers increased with incubation time for first hour then remained constant. Leveling of percent dimers was due to precipitation of protein (see *b*) and not to completion of cross-linking reaction. (*b*) Amount of protein on the gel decreased due to lysozyme precipitation by prolonged presence of glutaraldehyde.

cross-linking reaction time was held constant at 10 min. From Fig. 4, it is apparent that the number of aggregates did not change with changes in the aggregation time suggesting that the amount of time between addition of salt and cross-linking does not affect the types and numbers of aggregates. In fact, the average percentage of dimers was constant at 15 ± 2%. This implies that the aggregation reaction is faster than the cross-linking reaction.

The kinetics of the cross-linking reaction were studied by varying the amount of time the glutaraldehyde was allowed to react with the solution before the reaction was stopped with addition of hydrazine. The glutaraldehyde was added to the protein solution immediately after addition of NaCl. Final solution conditions were 1.5 mg ml^{-1} lysozyme and 7% NaCl, pH 4.0. It was found that the time allowed for the glutaraldehyde cross-linking reaction did increase the amount of aggregates for the first 3 h after which the number of aggregates began to level off (see Fig. 5*a*). This decrease was found to be because of less protein on the gel from precipitation of the protein by glutaraldehyde rather than completion of the cross-linking reaction. Fig. 5(*b*) shows the amount of soluble protein found in solution after exposure to glutaraldehyde for different lengths of time.

3.4. *Effect of salt type on the aggregation of lysozyme*

Since electrostatic interactions are proposed to be involved in the process of crystallization we decided to study the effect of different salts on the aggregation process. Because glutaraldehyde will react with any amine group we were not able to study ammonium salts or thiocyanate salts. Our first attempt was to scan salt concentrations for two salts, sodium chloride and sodium sulfate. Fig. 6 shows the effect of salt

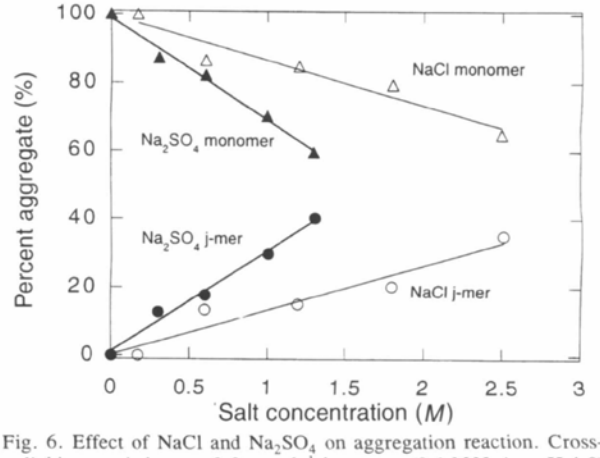


Fig. 6. Effect of NaCl and Na_2SO_4 on aggregation reaction. Cross-linking carried out at 2.8 mg ml^{-1} lysozyme (0.1 *M* NaAc, pH 4.0) with a 1000:1 molar ratio of glutaraldehyde to lysozyme. Curves correspond to: (△) NaCl monomers, (▲) Na_2SO_4 monomers, (○) NaCl total aggregates and (●) Na_2SO_4 total aggregates.

concentration on the total percentage of monomers and aggregates at a constant lysozyme concentration of 1.5 mg ml^{-1} (0.1 *M* NaAc, pH 4.0). Sodium sulfate produced more aggregates than NaCl at equimolar concentrations. A ratio of the slopes of the aggregate line for Na_2SO_4 is approximately three times that for NaCl.

4. Discussion

We have detected aggregates in medium to high ionic strength solutions using glutaraldehyde cross-linking and SDS–PAGE separation. It was found that the purified cross-linked dimers were still enzymatically active, however, the dimer was less active than the monomer. Thus, the bonds forced between molecules might be interfering with the active site of the molecule. This is not detrimental to the use of cross-linking as a means of quantifying aggregation and might be useful in identifying which residues on the lysozyme molecule are involved in cross-linking.

The most significant assumption we have made is that lysozyme is monomeric at 1% NaCl and pH 4.0. Thus, if no aggregates were seen in the cross-linked products under these conditions then there was no inter-aggregate cross-linking is occurring due to collisions. Once it was established that no aggregates were detected with our glutaraldehyde/protein molar ratio at 1% NaCl we went to medium and high salt concentrations where crystals or precipitate were likely to form. At 7% NaCl, pH 4.0 we detected aggregates at protein concentrations as low as 2 mg ml^{-1}. Since the reported solubility of lysozyme in 7% NaCl and pH 4.0 is 1.02 mg ml^{-1} (Cacioppo & Pusey, 1992) this represents a twofold supersaturated solution. Also, at 7% NaCl we did not see any aggregates below the solubility concentration. This contradicts some reports of aggregates in undersaturated solutions (Wilson & Pusey, 1992). An increase in the NaCl concentration resulted in aggregates at even lower protein concentrations. At the highest salt concentration and protein concentration studied we detected three aggregate species with molecular weight above the monomer. Their concentration profile is shown in Fig. 3(*b*). The largest aggregate detected was that of a tetramer and all intermediate species between monomer and tetramer were present. This suggests that the aggregates increase in size by addition of monomer units and that both odd-numbered and even-numbered species are present.

The molecular weights of the aggregates did not correspond to the multiple molecular weights of the monomer. Only intermonomer cross-links would have any effect on the molecular weight of the aggregate products. That the molecular weights are low suggests compact structures *versus* linear structures. The lower molecular weight of the dimer could result from multiple cross-links between protein molecules. The lower monomer molecular weight after cross-linking suggests a small degree of intramolecular cross-linking. The possibility of multiple cross-links between molecules and intramolecular cross-linking lowers the number of lysines available to form higher aggregates. This might explain the absence of oligomers larger than tetramers at the highest salt concentration of 15% NaCl. It has been suggested that octamers are the growth unit for lysozyme crystallization (Forsythe & Pusey, 1994). Yet octamers are noticeably absent in our work. Their absence might result from the fact that there are no free lysine residues available for intermolecular cross-linking. Li, Nadarajah & Pusey (1995) have suggested an aggregation pathway which includes octamers that follows the order of monomer > dimer > tetramer > octamer > sixteenmer. Our results show the pathway proceeds by monomer addition with a detectable amount of trimer present and termination with tetramer. Whether or not there are experimental reasons, such as intramolecular cross-linking, which prevent cross-linked octamers from forming remains to be seen. We are currently expanding these experiments to higher protein concentration to look for the larger aggregates.

Since chemical cross-linking with glutaraldehyde has been used to study the association kinetics of oligomeric systems (Hermann, Rudolph & Jaenicke, 1979), we decided to study the time dependence of aggregation. However, we found the aggregation to be unaffected by the length of time allowed between adding salt and fixation with glutaraldehyde which suggests that the aggregation reaction is faster than the cross-linking reaction (Fig. 4). This is further complicated by the fact that the number of aggregates was directly related to the amount of time the cross-linking agent was active before deactivation with hydrazine (Fig. 5). The reaction did not come to completion even after 3 h and resulted in precipitation of the protein with times greater than 3 h. The slow cross-linking reaction is believed to be a result of the acidic pH where glutaraldehyde is known to exist as cyclic polymers in solution (Hardy, Nicholls & Rydon, 1976).

The slow cross-linking reaction has implications in interpretation of the quantity of aggregates. We recognize that the cross-linking process is irreversible while the aggregation reaction might very well be reversible. In the presence of the cross-linker these short-termed aggregates would be fixed permanently. Because of the slow cross-linking reaction we must use long time periods to allow at least partial completion of the cross-linking reaction. Over the 10 min time period we are detecting a summation of the aggregates formed. However, as long as the cross-linking incubation time remains constant we can study the effect of salts on the association of molecules keeping in mind the quantity of aggregates will be lower than actual because the cross-linking reaction is not complete in the 10 min incubation time. The slow cross-linking reaction also prevents a

study of the time dependence of aggregation. Furthermore, it prevents the use of this approach to study the effect of temperature on aggregation since temperature differences will greatly effect the rate of the cross-linking reaction. We are currently trying to decrease the molar ratio of glutaraldehyde to protein which will enable the cross-linking reaction to reach completion before precipitation of the protein occurs. If this is successful then the temperature dependence of aggregation will be pursued.

One of our goals in this study was to compare the aggregate distributions of different inorganic salts. Initially, we studied NaCl and Na_2SO_4 at pH 4.0 (0.1 *M* NaAc). In a study of solubility measurements of lysozyme, Riès-Kautt & Ducruix (1989) were not able to crystallize the protein in the presence of sulfate ions irrespective of the cation used (NH_4^+, Na^+, K^+ or Li^+). They later obtained tetragonal crystals in H_2SO_4 and acidic pH (Riès-Kautt, Ducruix & Van Dorsselaer, 1994). Monoclinic lysozyme crystals have been obtained in the presence of 0.77 *M* Na_2SO_4 and 0.5 *M* NaAc (Steinrauf, 1959). Although sulfate ions were present, the high acetate concentration may have played a role in crystallization. At low acetate concentrations and acidic pH the use of NaCl and Na_2SO_4 allows for a comparison of aggregates which lead to crystals with those which lead to amorphous precipitate. We found that under equimolar conditions Na_2SO_4 produces 2–3 times the number of aggregates as NaCl (Fig. 6). This would indicate that the attraction between protein molecules is stronger in Na_2SO_4 than in NaCl. Considering that the ionic strength of a Na_2SO_4 solution is three times that of an equimolar NaCl solution this is not surprising. When corrected for ionic strength the number of aggregates for the two salts are equivalent for equivalent ionic strengths. This would suggest that shielding of the repulsive positively charged lysozyme molecules by the ionic character of the solution allows the attractive interaction resulting in aggregates. Clearly, more work needs to be carried out to determine if the identity of the anion effects the types and numbers of aggregates. In the future, we hope that knowledge of the types and numbers of aggregates under different solution conditions will allow us to explain why some conditions lead to crystals and others to amorphous precipitate.

5. Conclusions

Chemical cross-linking with glutaraldehyde has been used to study crystallizing lysozyme solutions at pH 4.0. The cross-linking reaction was found to be slow at this pH preventing a study of either the kinetics of the aggregation process or the effect of temperature on aggregation. The main results are summarized below.

No aggregates were detected in low-salt acidic solutions of lysozyme which suggest that no collisional cross-linking is occurring.

High ionic strength solutions of NaCl and Na_2SO_4 are highly aggregated the latter salt producing the largest number of aggregates.

Because of the slow cross-linking reaction the numbers of aggregates we measured are believed to be low since the reaction never reached completion.

The aggregates increase by addition of single units suggesting the aggregation pathway to be that of monomer addition.

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