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Supersaturated lysozyme solution structure studied by chemical cross-linking

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Glutaraldehyde cross-linking followed by separation has been used to detect aggregates of chicken egg-white lysozyme (CEWL) in supersaturated solutions. In solutions of varying NaCl content, the number of aggregates was found to be related to the ionic strength of the solution. Separation by SDS–PAGE showed that percentage of dimer in solution ranged from 25.3% for no NaCl to 27.1% at 15% NaCl, and the aggregates larger than dimer increased from 1.9% for no NaCl to 36.8% at 15% NaCl. Conversely, the percentage of monomers decreased from 72.8% without NaCl to 36.1% at 15% NaCl. Molecular weights by capillary electrophoresis (SDS–CE) were found to be multiples of the monomer molecular weights, with the exception of trimer, which indicates a very compact structure. Native separation was accomplished using size-exclusion chromatography (SEC) and gave a lower monomer concentration and higher aggregate concentration than SDS–CE, which is a denaturing separation method. Most noticeably, trimers were absent in the SEC separation. The number of aggregates did not change with increased time between addition of NaCl and addition of cross-linking agent when separated by gel electrophoresis (SDS–PAGE). The results suggest that high ionic strength CEWL solutions are highly aggregated and that denaturing separation methods disrupt cross-linked products.

1. Introduction

The very first step in protein crystallization is the establishment of supersaturation by addition of precipitating agent resulting in self-association of individual protein molecules. While many groups have studied the surface structure of proteins (Malkin *et al.*, 1995, 1996; Konnert *et al.*, 1994; Li *et al.*, 1999), our work focuses on the composition of the solution from which protein crystals grow. An important contribution of recent surface characterization work using atomic force microscopy (AFM) and its older sibling, electron microscopy, has been information concerning the incorporation of defects which plays a direct role in determining the quality of the crystal. For canavalin, Land *et al.* (1995) detected holes or hollow channels of the order of 2 μm in diameter. These holes were postulated to be caused by the incorporation of foreign particles adsorbed onto the crystal surface. This process was also observed by Durbin & Carlson (1992) and Durbin *et al.* (1993) by electron microscopy. Malkin *et al.* (1995) demonstrated incorporation of microcrystals onto the surface of protein and virus crystals by AFM. What was most interesting about this finding was that the microcrystals were completely swallowed up by the growing crystals. Unfortunately, no quantitative work has been done to confirm a relationship between crystal quality and foreign particle incorporation, although several studies are under way. It seems reasonable to propose that incorporation of foreign particles (*i.e.* dust or impurity) into the crystalline lattice would be detrimental to diffraction quality. Since the foreign particles originate from the supersaturated protein solution it is important that the solution structure be fully characterized. In fact, we suggest that nucleation debris (*i.e.* subcritical sized ordered aggregates of the protein of interest) could be incorporated more easily than foreign particles and the great abundance of these oligomers would increase the chance of their incorporation into the crystal lattice. These oligomers may or may not have crystallographic

structure. If they do not it is easy to justify the decrease in diffraction quality due to their incorporation into the lattice. However, if they do have crystallographic structure their improper orientation may lead to increased disorder in the same way that microcrystals are thought to be detrimental to crystal quality.

Evidence of oligomers in supersaturated lysozyme solutions comes from many sources including sedimentation equilibrium (Sophianopoulos & Van Holde, 1961, 1964), calorimetry (Banerjee *et al.*, 1975), nuclear magnetic resonance (Shindo *et al.*, 1977), light-scattering intensity (Pusey, 1991), dialysis kinetics (Wilson *et al.*, 1993) and neutron scattering (Boue *et al.*, 1993). Some of these studies were performed 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. A recent study on the solution aggregation properties of CEWL using pulsed-gradient spin-echo (PGSE) NMR has shown that CEWL aggregates in solutions of high NaCl concentration and that the process depends on solution conditions of pH and NaCl concentration (Price *et al.*, 1999). Carlsson *et al.* (2001) used Monte Carlo simulations to model the solution structure of lysozyme. Calculated structure factors and association constants agreed with experimental results. Li *et al.* (1995) have proposed that the aggregation proceeds by successive addition of monomers to form dimers, the addition of dimers to form tetramers and so forth. This model has been backed up experimentally by dialysis kinetics (Wilson *et al.*, 1996) and PGSE NMR (Price *et al.*, 1999).

Intermolecular covalent cross-linking of protein has been used to study protein–protein interactions (Fancy, 2000). Glutaraldehyde is the most widely used cross-linking agent because of its ability to cross-link the amino groups that are in high abundance in most proteins (Hermanson, 1996). While there are many newer bioconjugation reagents available our goal was to extend our previous work with glutaraldehyde which used a very narrow lysozyme concentration range (0–3 mg ml^{-1}; Wang *et al.*, 1996). That work indicated that aggregates increased by the addition of monomer units which does not agree with the model proposed by Li *et al.* (1995). In this paper we have extended the protein concentration range to 10 mg ml^{-1} at acidic pH. In addition we have separated the oligomers using native SEC. For comparison, we also used the denaturing separation methods of SDS–PAGE and SDS–CE. A difference in separated cross-linked product is reported between the native and denaturing separation methods.

2. Materials and methods

2.1. Materials

CEWL was purchased from Calbiochem (La Jolla, CA, USA). Buffer components, NaCl, glutaraldehyde [50%(*w*/*w*)] and anhydrous hydrazine were all purchased from Fisher Scientific (Fair Lawn, NJ, USA). Spectra/Por molecular porous membrane tubing 6000–8000 molecular-weight cutoff (MWCO) was from Spectrum Medical Industries, Inc. (Los Angeles, CA, USA). Pleated dialysis tubing 10000 MWCO was from Pierce (Rockford, IL, USA). Electrophoresis reagents were from Pharmacia Co. (Uppsala, Sweden). Blue Dextran was acquired from Sigma Chemical Co. (St Louis, MO, USA). Gel-filtration molecular-weight standards were from Bio-Rad Laboratories (Hercules, CA, USA).

2.2. Protein preparation

In order to remove small-molecule impurities in commercial proteins, dialysis was employed. 1 g CEWL was dissolved in about 8 ml of 0.1 *M* NaAc buffer pH 4.0. It was dialyzed three times against 350 ml of the same buffer in a sealed 6000–8000 MWCO membrane tubing overnight. The concentration of protein was determined at 280 nm using an absorption coefficient of 26.41 (Aune & Tanford, 1969). Before storage at 277 K, purified protein solutions and all the other solutions, including buffer and NaCl solutions, were passed through 0.2 µm disposable filters from Whatman Ltd (Ann Arbor, MI, USA).

2.3. Cross-linking

Different amounts of 25%(*w*/*w*) NaCl in 0.1 *M* NaAc pH 4.0 buffer solution were added to CEWL stock solution to yield the desired concentration. These solutions were immediately reacted with glutaraldehyde at a 500:1 molar ratio of glutaraldehyde to CEWL for 10 min. Hydrazine at a molar ratio of 10:1 to glutaraldehyde, was used to terminate the reaction. Dialysis three times against deionized water using 6000–8000 MWCO membranes was carried out overnight to remove NaCl and reaction products.

2.4. SDS–PAGE

Separation of aggregates was performed by SDS–PAGE on a PhastGel 8-25 gradient mini-slab gel with PhastGel SDS buffer strips and Coomassie blue stained according to the manufacturer's recommendations. Quantification of the bands on the gel was performed using *UN-SCAN-IT* software. Pharmacia's electrophoresis low-molecular-weight calibration kit was used as protein standards for molecular-weight determination of the aggregates.

2.5. SDS–CE

Capillary electrophoresis was performed on the Bio-Rad BioFocus 2000 (Bio-Rad Laboratories, Hercules, CA, USA) using the CE-SDS Protein Kit following manufacturer's recommendations with detection at 220 nm. An internal reference (benzoic acid, 1 mg ml^{-1}) was also added to the sample to ensure proper sample injection. The sample was applied to a fused silica capillary (24 cm × 50 µm) by pressure injection (5 p.s.i. for 8 s) and a constant voltage was applied at 15 kV normal polarity for 15 min. The capillary temperature remained constant at 293 K.

2.6. Size-exclusion chromatography (SEC)

SEC analysis used 1.0 ml injections onto a Bio-Sil 125 and Bio-Sil 250 used in tandem to achieve optimum results. Each column had a length of 300 × 7.8 mm, with a pore size of 5 µm. Detection was at a wavelength of 280 nm. Chromatogram quantitation was performed using Bio-Rad *EZ-Logic* software integration. The mobile phase used was 0.05 *M* Tris pH 8 with 0.60 *M* NaCl (Awadé & Efstathiou, 1999). Standard molecular-weight makers (Bio-Rad Laboratories) in the range 1350–158 000 Da. Thyroglobulin (670 000 Da) served as the column-void volume determinate. The calibration equation for the graph of $\log_{10}$(MW) *versus* retention time was found to be $y = -0.13x + 7.362$, with $R^2 = 0.980$. This calibration equation was used to determine the apparent molecular weight of the monomeric and cross-linked CEWL. Monomeric CEWL was injected five times at a concentration of 1.0 mg ml^{-1} (the inset in Fig. 4 is typical). The native molecular weight was found to be 14 192 ± 30 Da.

3. Results

3.1. SDS–PAGE

Glutaraldehye has been used extensively to study protein–protein interactions (Habeeb & Hiramoto, 1968; Schejter & Bar-Eli, 1970; Wang *et al.*, 1996; Yonath *et al.*, 1977). Our initial study used glutaraldehyde to study aggregation in the CEWL concentration range of 0–3 mg ml^{-1}, while the concentration of precipitant agent, NaCl, was varied from 0–15% using a fixed glutaraldehyde:protein molar ratio of 1000:1 (Wang *et al.*, 1996). In this study we expanded the protein concentration to 10 mg ml^{-1} and NaCl concentrations from 0 to 15% (0.1 *M* NaAc pH 4.0) which is closer to the concentrations commonly used for crystallization trials. At these protein concentrations it was necessary to reduce the glutaraldehyde:protein molar ratio to 500:1 to prevent precipitation. Following cross-linking the aggregates were separated on SDS–PAGE (Fig. 1) with Coomassie blue staining. Following analysis by densitometry, it was found that the percentage of dimer in solution ranged from 25.3% for no NaCl to 27.1% at 15% NaCl and the aggregates larger than dimer increased from 1.9% for no NaCl to 36.8% at 15% NaCl. Meanwhile, the percentage of monomers decreased from 72.8% for no NaCl to 36.1% at 15% NaCl (Table 1).

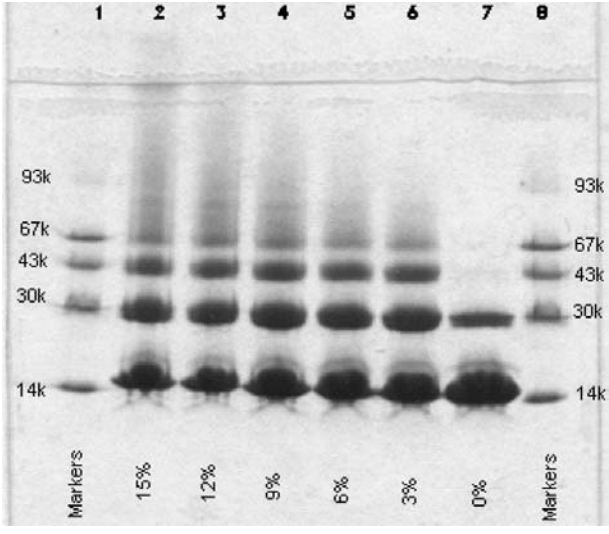

Figure 1
SDS–PAGE with Coomassie blue staining of cross-linked product at different concentrations of NaCl. Lanes are (1) molecular-weight markers, (2) 15%, (3) 12%, 4) 9%, (5) 6%, (6) 3% of NaCl, (7) control with no NaCl added, (8) molecular-weight markers. CEWL concentration was 10 mg ml^{-1} buffered at pH 4.0 (0.1 *M* NaAc buffer). The ratio of glutaraldehyde to protein was 500:1. Amount of protein loaded onto gel was 4 µg per lane.

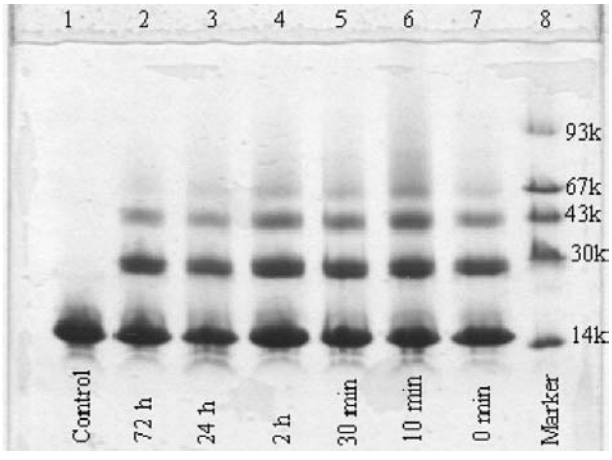

Figure 2
SDS–PAGE with Coomassie blue staining of cross-linked product with different aggregation times. Lanes are (1) 72 h, (2) 24 h, (3) 2 h, (4) 30 min, (5) 10 min for aggregation time, (6) control with no glutaraldehyde, (7) molecular weight markers. CEWL concentration was 10 mg ml^{-1} , buffered at pH 4.0 (0.1 *M* NaAc buffer). NaCl concentration was 10%, ratio of glutaraldehyde to protein was 500:1. Amount of protein loaded onto gel was 4 µg per lane.

Table 1
Percentage of cross-linked CEWL aggregates determined by densitometry of the gel shown in Fig. 1.

NaCl (%)	Monomer (%)	Dimer (%)	>Dimer (%)
0	72.8	25.3	1.9
3	43.5	30.5	26.0
6	40.8	29.5	29.7
9	39.8	29.9	30.4
12	34.7	28.5	36.8
15	36.1	27.1	36.8

3.2. Time-dependence of aggregation

Given that supersaturated solutions lead to increased particle size, the time dependence of aggregation was followed by varying the amount of time between the addition of precipitant and addition of glutaraldehyde from immediately to 3 d. From the gel shown in Fig. 2, it is obvious that there is no increase in the number of aggregates over the time frame studied. Since SDS–PAGE is a denaturing method of separation it is possible that the cross-links are adversely affected.

3.3. SDS–capillary electrophoresis

In order to compare the number and quantity of aggregates between denaturing and native separations an analytical separation must be achieved. SDS–CE is similar to the gel electrophoresis in terms of sample preparation. However, it allows for peak integration which is much more quantitative than densitometry of gels bands. Fig. 3 is an electropherogram of cross-linked product at 3% NaCl and 7 mg ml^{-1} CEWL at pH 4.0 (0.1 *M* NaAc). Excellent resolution of the

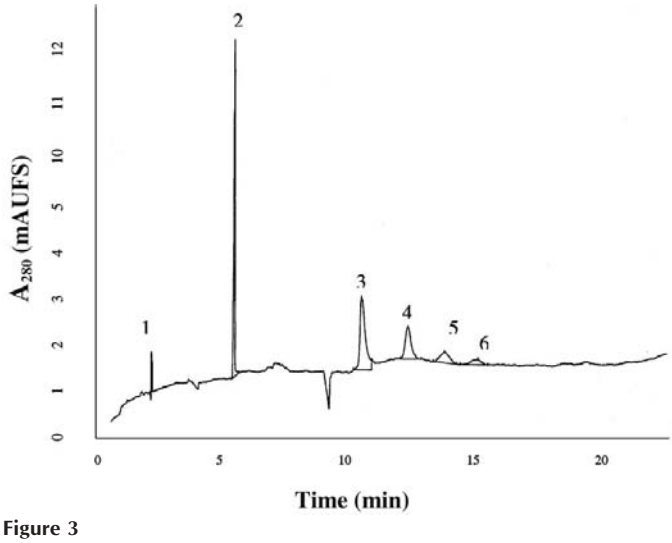

Figure 3
SDS–CE of cross-linked product at 3% NaCl and CEWL concentration of 7 mg ml^{-1} buffered at pH 4.0 (0.1 *M* NaAc buffer). The ratio of glutaraldehyde to protein was 500:1. Peak (1) is unretained electrophoretic buffer and (2) is benzoic acid added as an internal standard. Comparing to protein MW standards (run not shown) the molecular weights of cross-linked product were found to be to be (3) 14 108 Da, (4) 28 695 Da, (5) 35 352 Da and (6) 56 348 Da.

individual protein aggregates was obtained. Using molecular-weight standards the molecular weights of the aggregates were found to be 14.1, 28.7, 35.3 and 56.3 kDa for the monomer, dimer, trimer and tetramer, respectively. The ideal multiple molecular weights would be 14.4, 28.8, 43.2 and 57.6 K for the monomer, dimer, trimer and tetramer, respectively. The experimental values represent multiples of the monomer molecular weight of 1.0, 2.0, 2.5 and 4.0, respectively. The molecular weight of the trimer was lower than expected which suggests a compact structure instead of a linear structure. In general there was an increase in the percentage of aggregates larger than dimer with an increase in concentration of NaCl. Peak area integration showed the distribution of aggregates to be 59.77% monomer, 26.79% dimer, 8.84% trimer and 4.60% tetramer.

3.4. Size-exclusion chromatography

In order to study the solution behavior of our cross-linked aggregates, we decided to use SEC since it is a method by which separation is based on size, with the largest aggregates eluting first. Initial efforts were unsuccessful because lysozyme was found to exhibit anomalous SEC behavior suggesting interaction with the SEC column. Using a similar stationary Awadé & Efstathiou (1999) found that buffers containing less than 0.1 *M* NaCl led to retention of lysozyme on the column due to the high p*I* of lysozyme (p*I* = 10.7). For this reason we used the highest pH allowed for the columns (0.05 *M* Tris pH 8) and 0.6 *M* NaCl.

Fig. 4 shows a chromatogram of the same sample used in Fig. 3 (3% NaCl and 7 mg ml^{-1} CEWL at pH 4.0 in 0.1 *M* NaAc). The high NaCl concentration in our mobile phase assures the additional peaks seen in Fig. 4 are not due to ionic interactions with the stationary phase. Furthermore, as can be seen in the inset of Fig. 4, non-cross-linked monomeric lysozyme was found to give a single peak, which is further evidence that there is no interaction between lysozyme and the stationary phase using these conditions.

There were three aggregates detected with molecular weights of 12.9, 25.7 and 51.5 kDa, respectively. The 12.9 kDa molecular weight represents the monomer and is found to be significantly different than the 14.1 kDa from SDS–CE and SDS–PAGE ($p < 0.01$). Furthermore, it is 9.1% lower than the 14.2 kDa found in non-cross-linked solutions. The lower molecular-weight monomer suggest a significant degree of intramolecular cross-linking which may exert a physical constraint on the monomer thereby reducing the hydrodynamic volume and giving a smaller apparent molecular weight. However, SDS–CE and SDS–PAGE (Wang *et al.*, 1996) both gave monomer molecular weights of around 14.1 kDa.

Dividing the molecular weights of the aggregates by the native cross-linked monomer molecular weight gives multiples of 2 (1.99) and 4 (3.99). Integration of peak areas yields 52.53% monomer, 34.25% dimer and 13.23% tetramer. Comparison of the percent monomer by SEC with that found by SDS–CE showed a significant difference ($p < 0.05$). The absence of trimer is significant since it disagrees with the results seen by SDS–CE and SDS–PAGE.

Since NaCl is thought to induce supersaturation by increasing the ionic strength, we ran a control cross-linking reaction at 7 mg ml^{-1} CEWL and no NaCl. A control was run containing lysozyme with no glutaraldehyde and obtained one peak indicating a monomeric lysozyme with a molecular weight of 14.2 kDa (inset in Fig. 4). Cross-linking at 0% NaCl and 7 mg ml^{-1} is shown in Fig. 5. The first peak is a small amount of dimer with molecular weight of 25.5 kDa. The second peak is the monomer with molecular weight of 12.6 kDa. Dividing dimer molecular weight by the monomer molecular weight gives a ratio of 2.02. The low molecular weight of the monomer is an indication of intramolecular cross-linking. Integration of peak areas showed 2.33% dimer and 97.67% monomer.

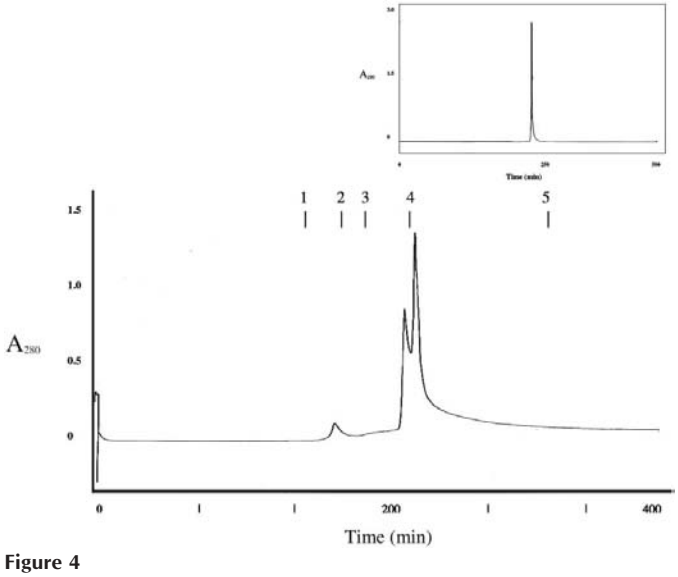

Figure 4
Native SEC of cross-linked product at 3% NaCl and CEWL concentration of 7 mg ml^{-1} buffered at pH 4.0 (0.1 *M* NaAc buffer). The ratio of glutaraldehyde to protein was 500:1. Peaks represent molecular weights of (1) 51.5 kDa (tetramer), (2) 25.7 kDa (dimer) and (3) 12.9 kDa (monomer). Noticeably absent is trimer. The mobile phase was 0.05 *M* Tris pH 8.0 plus 0.60 *M* NaCl, isocratic flow at 0.1 ml min^{-1}. Standards were run in the same mobile phase and their retention times, RT, and molecular weights marked as 1–5 were (1) thyroglobulin, 670 000 Da, RT 147.90 min, (2) IgG, 158 000 Da, RT 172.01 min, (3) ovalbumin, 44 000 Da, RT 192.44 min, (4) myoglobin, 17 000 Da, RT 229.43 min, (5) vitamin B_{12}, 1350 Da, RT 317.64 min. The inset figure is native SEC of 7 mg ml^{-1} CEWL as a control under the same conditions. The single peak represents monomeric lysozyme with molecular weight of 14 192 Da.

4. Discussion

Three analytical methods, SDS–PAGE, SDS–CE and SEC, were used to separate protein aggregates formed by chemical cross-linking with glutaraldehyde. The first method, SDS–PAGE, is fast and provides direct evidence of reaction products. However, it requires densito-

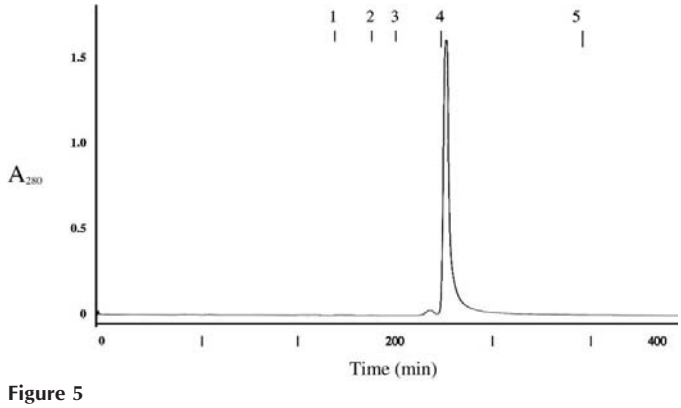

Figure 5
Native SEC of cross-linked product at 0% NaCl and CEWL concentration of 7 mg ml^{-1} buffered at pH 4.0 (0.1 *M* NaAc buffer). The ratio of glutaraldehyde to protein was 500:1. Peaks represent molecular weights of (1) 25.5 kDa (dimer) and (3) 12.6 kDa (monomer). Dimer to monomer molecular-weight ratio is 2.02. Peak area integration gives 2.33% dimer and 97.67% monomer. Mobile phase was 0.05 *M* Tris pH 8.0 plus 0.60 *M* NaCl, isocratic flow at 0.1 ml min^{-1}. Standards were run in the same mobile phase and their retention times, RT, and molecular weights marked as 1–5 were (1) thyroglobulin, 670 000 Da, RT 147.90 min, (2) IgG, 158 000 Da, RT 172.01 min, (3) ovalbumin, 44 000 Da, RT 192.44 min, (4) myoglobin, 17 000 Da, RT 229.43 min, (5) vitamin B_{12}, 1350 Da, RT 317.64 min.

metry for quantitation and is not very sensitive. SDS–CE is very sensitive and provides excellent resolution of the protein aggregates. It still requires SDS to impart a negative charge to produce the electro-osmotic flow through a sieving gel in the run buffer. Separation is based primarily on size because a sieving gel is added to the capillary. The only method that does not require denaturation is SEC, which gives a more accurate picture of the aggregate distribution in solution. SEC without cross-linking results in a single peak even at high salt concentrations (inset in Fig. 4). If higher order aggregates are present once they are separated from the monomer they dissociate during the separation to form more monomer to meet the equilibrium criteria. However, glutaraldehyde cross-linking adds a covalent bond to protein molecules whose ε-amino N atoms are 7.5 Å apart which implies that their C^{α} atoms are no more than 20 Å apart. Since only two cross-links have been observed in crystallographic data, it is thought that the cross-linking leads to flexible chains which would not be readily observable in difference maps (Yonath *et al.*, 1977).

Using the same cross-linking reaction mixture we obtained 52.53% monomer by SEC and 59.77% monomer by SDS–CE. This represents a 7.24% increase in the monomer when subjected to denaturation by SDS, β-mercaptoethanol and boiling. This indicates that some of the higher order aggregates are broken down to monomers during denaturation. Cross-links, just like disulfide bonds, restrain the natural unfolding and folding of the protein. However, it has been shown that even proteins with unreduced disulfide bonds can still be converted to random coils by denaturant (Tanford, 1968). Yonath *et al.* (1977) has shown that triclinic crystals of CEWL cross-linked with glutaraldehyde experienced a sharp volume increase and loss of X-ray pattern when treated with high concentrations of SDS. Since monomers are being produced during denaturation, the cross-linking reagent appears to be cleaved by reduction. We also lowered the ratio of glutaraldehyde:protein from 1000:1 in our previous work to 500:1 in this work. Lower concentrations of glutaraldehyde have been shown to result in less stable cross-links (Briand *et al.*, 1985).

More importantly, there was no trimer detected by SEC. Li *et al.* (1995) has suggested a possible pathway for crystallization building which includes octamers that follow the order of monomer $\rightarrow$ dimer $\rightarrow$ tetramer $\rightarrow$ octamers. Using SEC we see evidence of the first three species in supersaturated CEWL solutions, which is in agreement with this model. However, octamers are noticeably absent. Their absence may be due to the fact that all of the free lysine residues are saturated at the tetramer stage. Evidence for lack of free lysines is further supported by the low monomer molecular weight after cross-linking as determined by SEC (Fig. 5), which suggests a good deal of intramolecular cross-linking.

All three separation methods show direct evidence of dimers in the absence of NaCl (CE–SDS not shown, Fig. 1 lane 7 and Fig. 5). This is in disagreement with light-scattering studies (Bruzzesi *et al.*, 1965) and dialysis kinetics (Wilson & Pusey, 1992), which have shown CEWL to be monomeric in the absence of NaCl. This dimer is formed during the cross-linking process and is not the naturally occurring dimer of CEWL reported by Back (1984) since non-cross-linked CEWL was monomeric (inset in Fig. 4). One explanation for the formation of such dimers is the polymerization of glutaraldehyde. At high glutaraldehyde concentrations the formation of nonspecific inter-aggregate cross-links can occur which could lead to dimer formation even at low ionic strength. An increase in the chain length of a glutaraldehyde cross-link could potentially join proteins quite far apart from each other. NMR studies have shown that commercial glutaraldehyde solutions contain the dialdehyde in equilibrium with its cyclic monohydrate and open-chain monohydrate and dialdehyde (Hardy *et al.*, 1969). When the solution is near neutral or alkaline, the dialdehyde undergoes an intermolecular aldol condensation which is followed by dehydration to give α-β-unsaturated aldehyde polymers, $OHC-CH_2-[CH_2]_2-[CH{=}C(CHO)-[CH_2]_2]_n-CH{=}C(CHO)-[CH_2]_2CHO$ (Monsan *et al.*, 1975; Richards & Knowles, 1968). Commercial glutaraldehyde solutions of 25% at pH 3, have been found to contain 79% water, 3% glutaraldehyde and 18% derivatives of higher molecular weight (Hardy *et al.*, 1969). The higher molecular-weight derivatives of glutaraldehyde can react with proteins either in polymer form or by depolymerization (Monsan *et al.*, 1975). The polymerization of glutaraldehyde must be considered when interpreting the percent aggregates at higher ionic strength as well as the dimer formation at low ionic strength where lysozyme is known to be monomeric. It is possible that high ionic strength solutions increase the polymerization of glutaraldehyde, allowing higher aggregate concentrations. However, Ranatunga *et al.* (1999) used glutaraldehyde to cross-link high-mobility group-1 protein and reported that the extent of cross-linking was not affected by a 30-fold increase in salt concentration (from 4 to 120 m*M*). Because of the tendency to polymerize, glutaraldehyde cannot be used to determine intermolecular bond distances like more specific protein cross-linking agents. Also, to prevent polymers of glutaraldehyde from participating in the cross-linking it is important to keep the glutaraldehyde concentration relatively low. The age of the glutaraldehyde solution has been found to affect the extent of polymerization (Hermanson, 1996). For this reason, we used fresh glutaraldehyde solutions and stored the glutaraldehyde under nitrogen.

SDS–PAGE was used to separate cross-linked product with different amounts of time between addition of salt and the start of cross-linking. One would expect to see a gradual increase in particle size with time. However, there was no difference seen in the percentage and types of aggregates. Given the results of SEC it is now clear that SDS–PAGE does not give an accurate picture of the aggregate distribution. Larger aggregates are broken up into smaller ones during the denaturation process. Therefore, future work will focus on following the time-dependence of aggregation using native SEC for separation.

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