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Sedimentation Equilibrium Study of the Interaction between Egg White Lysozyme and Ovomucin

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Molecular weight distribution determination using sedimentation equilibrium ultracentrifugation was applied for investigating the interaction of lysozyme with native and reduced ovomucins. This method was found to be more sensitive and reliable than the earlier methods measuring precipitates or turbidity. The interaction seemed to be typically electrostatic: favored by lower temperatures and ionic strengths but decreased by acetylation of lysozyme. Removal of sialic acid residues from ovomucin had little effect. The extent of interaction of lysozyme with native ovomucin in 1:1 (w/w) mixtures was 20–30%, considerably lower than 70–80% with reduced, alkylated ovomucin at an ionic strength of 0.07. However, at an ionic strength of 0.13 the extent of interaction was about 6% with no difference between native and reduced, alkylated ovomucins. On the basis of these results using experimental conditions closer to those of natural egg white than have been previously reported, the ovomucin–lysozyme interaction is unlikely to be a cause of egg white thinning.

A number of workers have suggested that protein–protein interactions between ovomuucin and lysozyme are intimately involved in the maintenance of the gel structure of thick egg white and the process of egg white thinning (Kato and Sato, 1972; Kato et al., 1970a,b, 1971, 1975, 1976; Robinson, 1972; Robinson and Monsey, 1972). Attempts in the past to quantitate the extent of this interaction under varying conditions of pH and ionic strength have been based upon the observation that lysozyme and ovomucin will interact to form an insoluble complex (Dam, 1971; Dam and Bennett, 1963; Kato and Sato, 1972; Kato et al., 1971, 1975, 1976; Robinson, 1972; Robinson and Monsey, 1969). Detailed measurements of the extent of interaction have been obtained by using turbidimetric measurements at 450 nm (Robinson and Monsey, 1969, 1972), 550 nm (Kato et al., 1975, 1976), and 600 nm (Kato and Sato, 1972; Kato et al., 1971) of mixtures of lysozyme and reduced ovomucin. However, since not all interaction products could cause turbidity, these data may not be reliable.

Sedimentation equilibrium ultracentrifugation is more suitable for the study of macromolecule–macromolecule interactions (Howlett and Nichol, 1973). Molecular weight distributions of interacting proteins calculated by multiple regression analysis of sedimentation equilibrium data has recently been used to study the interaction of α_{s1}- and κ-caseins (van de Voort et al., 1979). An advantage of this technique over turbidimetric measurements is that soluble protein–protein interaction products can be detected. Moreover, the use of a UV scanning system provides a direct measure of protein concentration. Therefore, this method may be able to provide direct evidence of a lysozyme–ovomucin interaction.

Ovomucin is a complex of at least two distinct glycoproteins, α- and β-ovomucins (Hayakawa and Sato, 1976; Kato and Sato, 1971, 1972; Kato et al., 1971; Robinson and Monsey, 1971, 1975), and the interaction of lysozyme with β-ovomucin has been reported to be much stronger than that with α-ovomucin. Furthermore, it has been reported that the interaction is electrostatic in nature, involving the negative charges of the terminal sialic acid residues in ovomucin and the positive charges of the lysyl ϵ-amino groups in lysozyme (Kato et al., 1975, 1976). Since these determinations were made by using turbidimetric methods,

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it seems necessary to confirm them by using sedimentation equilibrium ultracentrifugation as a direct measure of protein–protein interactions.

It would also be interesting to see if there is any difference in the extent of interaction of lysozyme with reduced ovomucin compared to native ovomucin, in view of their large difference in molecular weight (Hayakawa and Sato, 1976; Robinson and Monsey, 1971, 1975; Tomimatsu and Donovan, 1972).

This paper describes the heterogeneous associations of lysozyme with reduced and native ovomucins as studied by using sedimentation equilibrium ultracentrifugation, the nature of the interaction, and the binding groups involved.

EXPERIMENTAL SECTION

Materials. Unless otherwise specified, all chemicals were reagent grade or the highest quality provided by the suppliers and were used without further purification. Neuraminidase (*Clostridium perfringens*, type IV) and *N*-acetylneuraminic acid (*Escherichia coli*) were purchased from Sigma Chemicals, St. Louis, MO. All eggs were collected from hens within 24 h after laying, and the shells were sterilized with 70% ethanol.

Preparation of Lysozyme. Lysozyme was prepared from fresh hen's egg white by the direct crystallization method (Alderton and Fevold, 1946), recrystallized 5 times, and lyophilized.

Preparation of Reduced Ovomucin. Thick egg white was separated from thin white (Kato et al., 1970a) and blended for 1 min at the slowest speed in a Sorval Omni-mixer (Ivan Sorvall, Norwalk, CO). Ovomucin was isolated as an insoluble gel following dilution of the blended thick white with 4% KCl according to the method of Kato et al. (1970b) and then reduced, and thiol groups were alkylated to prevent reoxidation, according to the method of Robinson and Monsey (1971). Urea substituted for guanidine hydrochloride. Following alkylation, reduced ovomucin was desalted by exhaustive dialysis at 4 °C against distilled water containing 0.02% sodium azide.

Preparation of Native Ovomucin. According to the method of Young and Gardner (1972), egg white diluted with 0.05M Tris-HCl buffer, pH 8.6, containing 0.85% NaCl, was blended twice and applied to a Sepharose 4B column (2.5 × 40 cm). The column was eluted with the same buffer at a flow rate of 13 mL/h, and 3.5-mL fractions were collected and analyzed for protein by measuring A_{280nm} and for sialic acid by the method of Warren (1959). Ovomucin eluted in the void volume was dialyzed at 4 °C against the centrifugation buffer. The ovomucin samples prepared here and above were electrophoretically pure (Kato et al., 1971; Young and Gardner, 1972).

Acetylation of Lysozyme. The free amino groups of lysozyme were acetylated according to the method of Yamasaki et al. (1968) as modified by Kato et al. (1975). The extent of amino group modification, determined spectrophotometrically according to the method of Haynes et al. (1967), was 100%.

Enzymatic Removal of Sialic Acid from Ovomucin. To a 2-mL solution of reduced or native ovomucin in 0.03 M sodium phosphate buffer, pH 6.9, containing 0.02% sodium azide, 1.85 μg of neuraminidase (EC 3.2.1.18, 4.2 units/mg) was added. Incubation was carried out for 22 h at 30 °C, and the extent of removal of sialic acid was determined by comparison with the total sialic acid released following acid hydrolysis with 0.1 N sulfuric acid at 80 °C for 1 h, according to the method of Warren (1959).

Ovomucin–Lysozyme Interaction. Sedimentation equilibrium ultracentrifugation was used to detect heterogeneous protein–protein associations of the type A + B = C according to the method described by van de Voort et al. (1979). The four-cycle computation with 8 data points each was performed, thus giving a total of 32 points. The interval index chosen was from 1.8 to 2.14, covering various molecular weight ranges between 10 000 and 6.6 million. The most frequently used index was 2.0 to cover the range up to M_r 2.5 million.

Separate exhaustive dialysis of proteins against phosphate (NaH_2PO_4, Na_2HPO_4) centrifugation buffer, pH 6.9, containing 0.02% sodium azide, preceded all centrifugation runs. Centrifugal runs were performed at 20 ± 0.5 °C, unless otherwise specified. Dialyzed lysozyme and ovomucin solutions were diluted such that absorbances at 280 nm were approximately 0.30 and then mixed in an ovomucin to lysozyme volume ratio of 1:4 prior to ultracentrifugation. Correcting for the UV absorbtivity difference between lysozyme and ovomucin, $A^{1\%}_{280nm,1cm}$ = 7.0 and 26.5 for ovomucin and lysozyme, respectively, gives weight to weight ratio of approximately 1:1. This ratio was chosen because an insoluble lysozyme–ovomucin aggregate forms in a mixture of both proteins where there is an excess of lysozyme (Kato et al., 1975; Robinson, 1972). It has been calculated that 1.97 g of lysozyme are required to cross-link 2 g of ovomucin at pH 7.4 (Robinson, 1972).

The effect of ionic strength on the interaction was investigated at ionic strengths of 0.13 and 0.07, pH 6.9, at 20 °C. The effect of temperature on the interaction was investigated by conducting equilibrium runs at 20 ± 0.5 and 3 ± 0.5 °C. For molecular weight distribution determination, the partial specific volume of lysozyme was taken as 0.726 mL/g (Deonier and Williams, 1970). A partial specific volume of 0.662 mL/g was calculated for ovomucin according to the method of Howlett and Nichol (1973) using values of 0.701 and 0.649 for α- and β-ovomucins, respectively (Robinson and Monsey, 1972), assuming that ovomucin consists of $(\alpha\text{-ovomucin})_n$:$(\beta\text{-ovomucin})_n$, where n is a positive whole number.

RESULTS

The effect of ionic strength on the reduced ovomucin–lysozyme interaction was investigated at pH 6.9. The molecular weight distribution patterns computed from sedimentation equilibrium data are shown in Figures 1–3. In Figure 1, it is observed that the area under the lysozyme peak (A_r) in the lysozyme-reduced ovomucin mixtures diminishes with decreasing ionic strength of solutions. Since the A_r in the molecular weight distribution patterns is approximately proportional to the concentration of that molecular weight species, the extent of interaction is calculated as the percent lysozyme that forms a complex with ovomucin and sediments to the cell bottom. It is the percentage decrease in the area under the lysozyme peak in the molecular weight distribution of mixtures (panels B and C) relative to the area under the lysozyme peak from the cell containing lysozyme only (panel A). The values are 5.6% and 70% at ionic strengths of 0.13 and 0.07, respectively (Table I). It should be noted that only a lysozyme peak (M_r 14 000) is seen in the molecular weight distribution patterns of these mixtures because the much larger molecules of reduced ovomucin and reduced ovomucin–lysozyme interaction products sediment to the cell bottom at the high equilibrium speed required to obtain a stable lysozyme pattern.

So that further information on this interaction, especially on changes in α- and β-ovomucins, could be obtained, the experiments were repeated by using slower rotor speeds. These results are shown in Figures 2 (ionic strength 0.13) and 3 (ionic strength 0.07). Panel B of

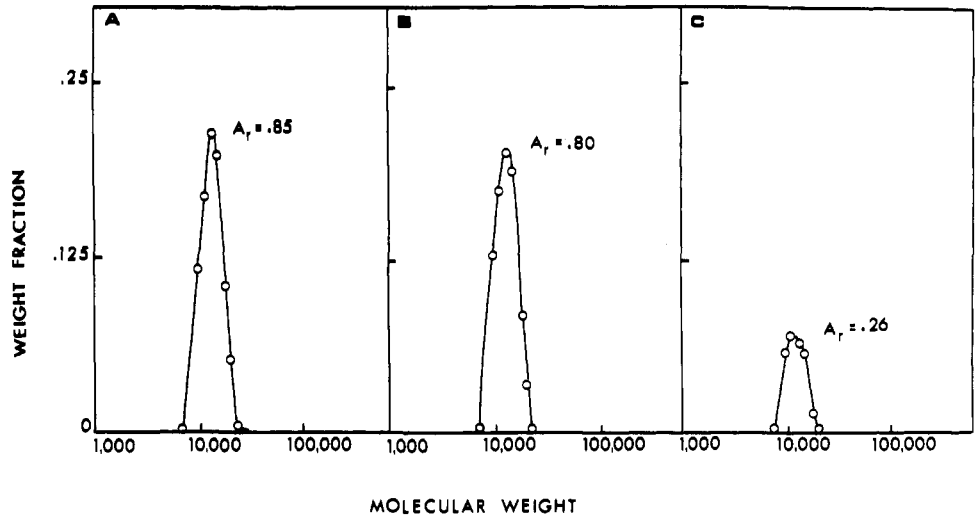

Figure 1. Molecular weight distribution of an RA ovomucin complex–lysozyme mixture at ionic strengths of 0.13 and 0.07 (A) Lysozyme at an ionic strength of 0.13. Initial absorbance, 0.298; rotor speed, 19 329 rpm. (B) RA ovomucin complex–lysozyme (1:1) at an ionic strength of 0.13. Initial absorbance, 0.298; rotor speed, 19 329 rpm. (C) RA ovomucin complex–lysozyme mixture (1:1) at an ionic strength of 0.07. Initial absorbance, 0.300; rotor speed, 19 944 rpm. The lysozyme peak only appears; other peaks are sedimented to the cell bottom. RA ovomucin = reduced, alkylated ovomucin. A_r = the area of lysozyme peak calculated as the total of weight fraction values within the peak; the A_r values should theoretically be 1.0 if no loss in lysozyme occurred during ultracentrifugation.

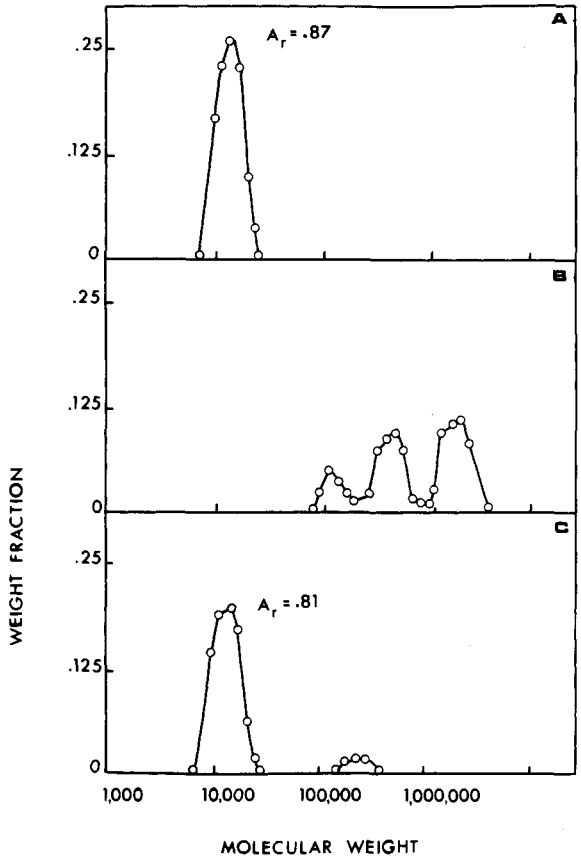

Figure 2. Molecular weight distribution of an RA ovomucin complex–lysozyme mixture at an ionic strength of 0.13. (A) Lysozyme. Initial absorbance, 0.300; rotor speed, 19 939 rpm. (B) RA ovomucin complex. Initial absorbance, 0.300; rotor speed, 9866 rpm. (C) RA ovomucin complex–lysozyme mixture (1:1). Initial absorbance, 0.298; rotor speed, 9866 rpm.

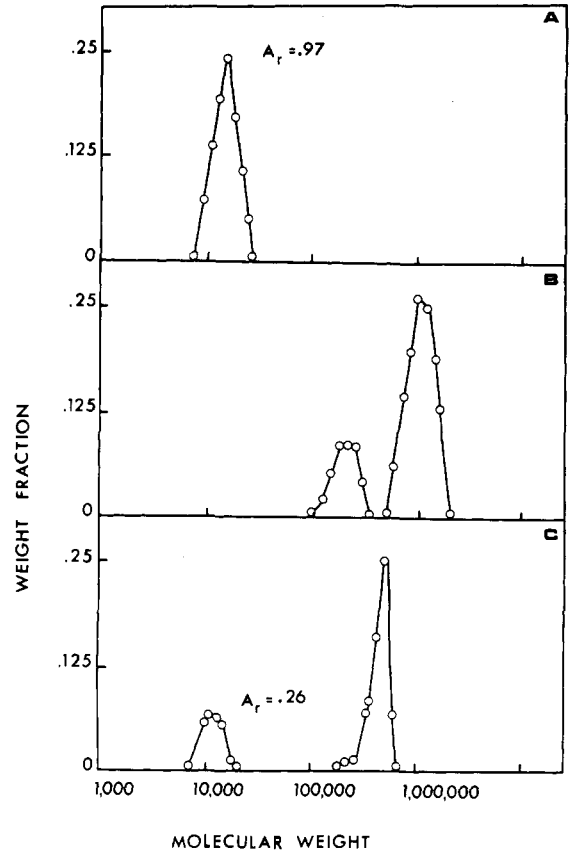

Figure 3. Molecular weight distribution of an RA ovomucin complex–lysozyme mixture at an ionic strength of 0.07. (A) Lysozyme. Initial absorbance, 0.300; rotor speed, 19 944 rpm. (B) RA ovomucin complex. Initial absorbance, 0.303; rotor speed, 9974 rpm. (C) RA ovomucin complex–lysozyme mixture (1:1). Initial absorbance, 0.305; rotor speed, 9974 rpm.

Figure 2 shows a trimodal distribution of reduced ovomucin, consisting of α-ovomucin peaks (M_r 116 000 and 494 000) and a β-ovomucin peak (M_r 1 113 000). For the reduced ovomucin–lysozyme mixture (panel C) a decrease in the lysozyme and α-ovomucin peaks and a complete disappearance of the β-ovomucin peak are observed. The extent of interaction of lysozyme is 6.9%. At a lower ionic strength of 0.07 (Figure 3, panel B), reduced ovomucin consists of a single α-ovomucin peak (M_r 221 000) and a β-ovomucin peak (M_r 1 149 000). For the reduced ovomucin–lysozyme mixture (panel C) complete disappearance of the β-ovomucin peak can be seen as well as the

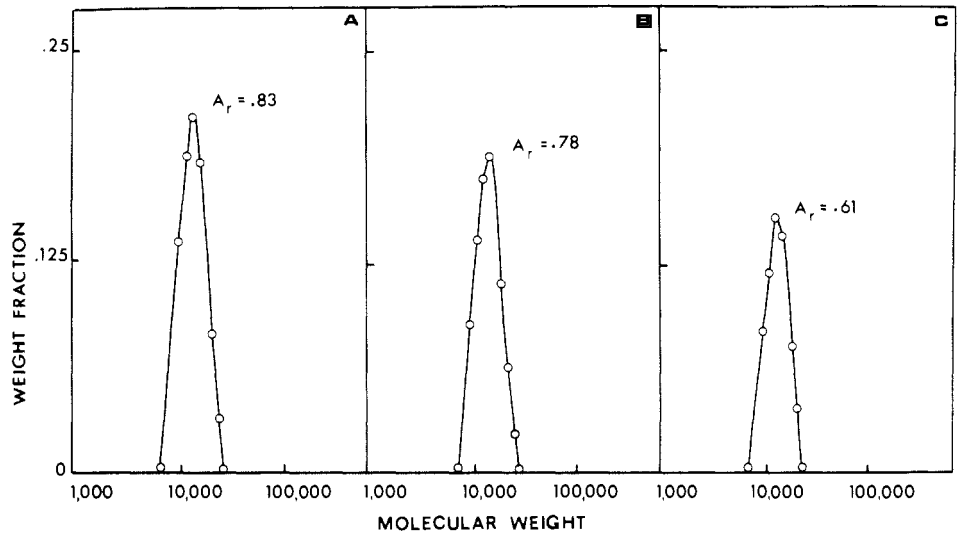

Figure 4. Molecular weight distribution of a native ovomucin–lysozyme mixture at ionic strengths of 0.13 and 0.07. (A) Lysozyme at an ionic strength of 0.13. Initial absorbance, 0.305. (B) Native ovomucin–lysozyme mixture (1:1) at an ionic strength of 0.13. Initial absorbance, 0.300. (C) Native ovomucin–lysozyme mixture (1:1) at an ionic strength of 0.07. Initial absorbance, 0.298; rotor speed, 31 908 rpm. The lysozyme peak only appears; other peaks are sedimented to the cell bottom.

Table I. Extent of Interaction of Lysozyme[a]

	reduced ovomucin at ionic strength		native ovomucin at ionic strength	
treatment	0.13	0.07	0.13	0.07
untreated control	5.6	70.0	5.7	27.0
acetylated lysozyme		10.6		6.8
asialoovomucin		76.0		19.5
asialo control		72.0		19.5
temperature				
20 °C	6.8	77.0		
3 °C	6.4	100.0		

[a] Calculated as the decrease (percent) of the lysozyme peak for comparison.

appearance of an intermediate peak (M_r 461 000) that may represent an interaction product between α-ovomucins and lysozyme. The extent of interaction of lysozyme with reduced ovomucin at an ionic strength of 0.07 is 73%.

The effect of ionic strength on the interaction of lysozyme with native ovomucin is shown in Figure 4. It was not possible to obtain readable molecular weight distribution patterns for native ovomucin in these mixtures because even at the slowest attainable rotor speed of about 4000 rpm the large ovomucin molecules and ovomucin–lysozyme interaction products rapidly sedimented to the cell bottom. Thus, an equilibrium speed of about 31 000 rpm was chosen to examine the lysozyme component only. The extents of interaction with lysozyme were 5.7% and 27% at ionic strengths of 0.13 and 0.07, respectively (Table I). At an ionic strength of 0.07, native ovomucin interacts with lysozyme about 0.4 times as strongly as reduced ovomucin.

Acetylation of the seven free amino groups in lysozyme markedly decreased the lysozyme–ovomucin interaction. At pH 6.9, ionic strength 0.13, the extent of lysozyme-reduced ovomucin interaction is 70% and that of the lysozyme–native ovomucin interaction is 27%; acetylation decreased this to 10.6% and 6.8%, respectively (Table I).

The effect of enzymatic removal of sialic acid residues from reduced ovomucin on the interaction with lysozyme at an ionic strength of 0.07 is shown in Figure 5. Panel B shows the molecular weight distribution pattern for the lysozyme-reduced ovomucin mixture, yielding a bimodal distribution consisting of lysozyme peak and an α-ovomucin peak (M_r 252 000) with the β-ovomucin–lysozyme complex having sedimented to the cell bottom. Panel C shows the molecular weight distribution pattern for the mixture containing reduced asialoovomucin, yielding a similar bimodal distribution, the asialo-β-ovomucin having

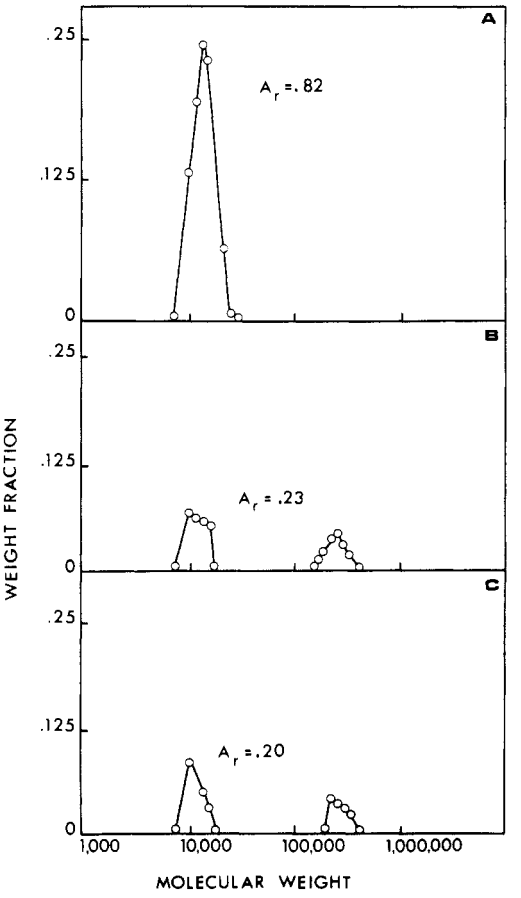

Figure 5. Molecular weight distribution of an RA asialoovomucin complex–lysozyme mixture at an ionic strength of 0.07. (A) Lysozyme. Initial absorbance, 0.297; rotor speed, 35 235 rpm. (B) RA ovomucin complex–lysozyme mixture (1:1). Initial absorbance, 0.298; rotor speed, 9263 rpm. (C) RA asialoovomucin complex–lysozyme mixture (1:1). Initial absorbance, 0.300; rotor speed, 9263 rpm.

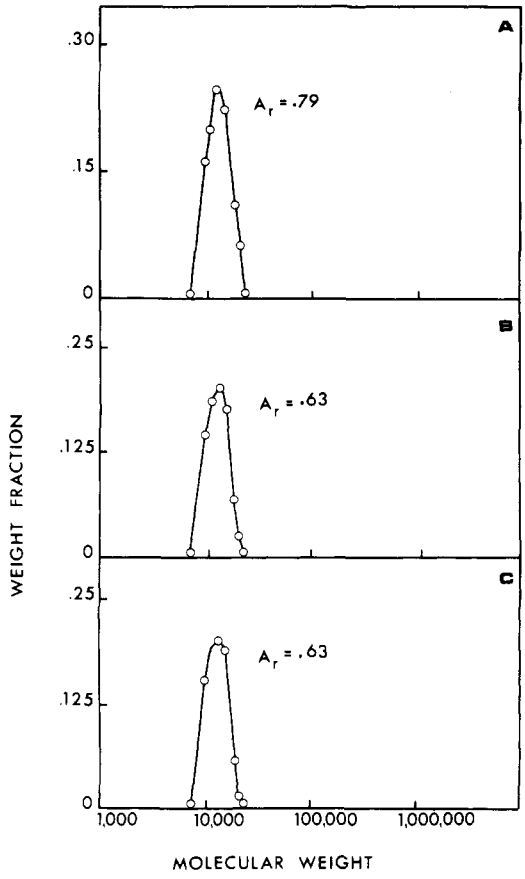

Figure 6. Molecular weight distribution of a native asialoovomucin–lysozyme mixture at an ionic strength of 0.07. (A) Lysozyme. Initial absorbance, 0.300. (B) Native ovomucin–lysozyme mixture (1:1). Initial absorbance, 0.300. (C) Native asialoovomucin–lysozyme mixture (1:1). Initial absorbance, 0.300; rotor speed, 35 798 rpm. The lysozyme peak only appears; other peaks are sedimented to the cell bottom.

sedimented to the cell bottom probably as an interaction product with lysozyme. Little or no proteolysis by contaminating enzymes is indicated by the similarity in the molecular weight distribution patterns of untreated and neuraminidase-treated, reduced ovomucins.

Removal of sialic acid from the native ovomucin did not affect the ovomucin–lysozyme interaction as shown in Figure 6.

With regard to the effect of temperature difference on the interaction of lysozyme with reduced ovomucin, it was found that a more extensive interaction was observed at 3 °C than 20 °C at an ionic strength of 0.07 but not at an ionic strength of 0.13 (Table I).

DISCUSSION

It has been shown by direct measurement of protein concentrations that the interaction of lysozyme with reduced and native ovomucins is electrostatic in nature, judging by the dependence of the interaction on ionic strength, temperature, and acetylation of lysozyme, which confirms earlier results (Kato and Sato, 1972; Kato et al., 1975, 1976; Robinson and Monsey, 1969). The sedimentation equilibrium technique is more sensitive and reliable than turbidimetric methods. The latter failed to detect an interaction in reduced ovomucin–lysozyme mixtures at an ionic strength of 0.13 and in mixtures of acetylated lysozyme with reduced ovomucin at an ionic strength of 0.07 (Kato et al., 1975). Furthermore, the interacting components of reduced ovomucin were identified by using sedimentation equilibrium, revealing a stronger interaction of lysozyme with β-ovomucin than with α-ovomucin (Figures 2 and 3). Kato et al. (1975) have reported that the interaction of purified β-ovomucin (F-ovomucin) was much stronger than that with purified α-ovomucin (S-ovomucin) in 0.07 M phosphate, pH 5.4. However, this pH is much lower than the in vivo pH of fresh egg white of approximately 7.4–7.8.

Self-association of reduced α-ovomucin at low ionic strength was observed (Figure 3, panel B). Robinson and Monsey (1971) have reported a self-association of reduced α-ovomucins at pH 7.5. The ionic strength dependent interaction between α-ovomucins at pH 6.9 may be electrostatic, although van der Waals forces and hydrogen bonds may also be involved (Waugh, 1954). Interestingly, an interaction between α-ovomucin and β-ovomucin is postulated since the peak corresponding to β-ovomucin is increased greatly at lower ionic strengths (Figures 2B and 3B). This is supported by the fact that the molecular weight of β-ovomucin is considerably greater than that reported by Robinson and Monsey (1971) in the presence of guanidine hydrochloride. The relationship between self-association of α-ovomucins and α,β-ovomucin interaction to the structure of thick egg white and the mechanism of egg white thinning needs further investigation.

There have been no previous reports on the interaction of lysozyme with native ovomucin, that is, ovomucin solubilized at neutral pH without chemical modification. It was found that native ovomucin interacts with lysozyme but to a lesser extent than does reduced ovomucin, particularly at low ionic strengths. It is known that native ovomucin consists of aggregated and polymerized molecules of interacting α- and β-ovomucins cross-linked by specific bonds (Adachi et al., 1973; Hayakawa and Sato, 1976) and that reduced ovomucin is a heterogeneous mixture of α- and β-ovomucins (Kato et al., 1971; Robinson and Monsey, 1971; Tomimatsu and Donovan, 1972). Since it has been reported that the conformation of the ovomucin polypeptide chain is not altered by reduction (Donovan et al., 1970), it is proposed that the greater interaction of lysozyme with reduced ovomucin is due to exposure of the carboxyl side chains, which contain the lysozyme binding sites, upon dissociation of ovomucin into α and β subunits by reduction of disulfide bonds. Supporting this premise are the observations that Pronase- or trypsin-digested ovomucin interacts more strongly with lysozyme than untreated ovomucin (Kato et al., 1975, 1976) and that the carbohydrate side chains of ovomucin seem to be concentrated along portions of the protein core (Kato et al., 1973). Surface charge measurements of reduced vs. native ovomucin may confirm this proposal.

The isoionic pH of lysozyme is 10.7 (Osuga and Feeney, 1977) whereas that of ovomucin is 4.5–5.0 (Donovan et al., 1970); hence, at pH 6.90 lysozyme will carry a net positive charge and ovomucin a net negative charge. Lysozyme has six lysyl ε-amino groups and an α-amino group (Canfield, 1963) carrying positive charges at pH 6.90, and these groups can be acetylated, resulting in a decrease in the net positive charge. Conversely, the negatively charged sialic acid residues of ovomucin can be removed, resulting in a decrease in net negative charge. The effects of these modifications on the electrostatic ovomucin–lysozyme interaction were studied. The extent of interaction of lysozyme with both reduced and native ovomucins was greatly decreased, but not completely abolished, following 100% modification of free amino groups in lysozyme. Hence, these groups are essential for the interaction. Kato et al. (1975, 1976) also showed that the lysozyme-reduced ovomucin interaction was markedly diminished by acety-

lation. However, the interaction was completely inhibited when only six amino groups were modified. Complete removal of sialic acid residues had little effect on the interactions of lysozyme with reduced or native ovomucins. Dam and Bennett (1963) and Dam (1971) also reported that enzymatic removal of sialic acid residues from ovomucin had little effect on lysozyme "binding". Thus, there must be sufficient negatively charged polar groups still present following asialation.

It is known that ovomucin contains ester sulfate, which would be negatively charged at pH 6.90, and that the ratio of sialic acid to ester sulfate is approximately 1:1 (Donovan et al., 1970; Kato and Sato, 1971; Robinson and Monsey, 1971). Since β-ovomucin contains sialic acid and ester sulfate whereas α-ovomucin does not (Kato et al., 1972, 1973) and since there is a preferential interaction of lysozyme with β-ovomucin, it is likely that the net negative charge of the ovomucin molecule, which originated from ester sulfates and to a lesser extent from sialic acid, is important for the electrostatic ovomucin–lysozyme interaction. Other acidic groups such as the carboxyl groups of glutamic and aspartic acids may also react with lysozyme (Robinson, 1972). However, this seems unlikely as α-ovomucin contains a greater number of these groups than does β-ovomucin. It is possible that, in addition to electrostatic interactions, a more specific type of interaction between the *N*-acetylglucosamine groups of β-ovomucin and the active site on the lysozyme is involved (Howlett and Nichol, 1973) as β-ovomucin contains 1.6 times as much *N*-acetylglucosamine as does α-ovomucin (Smith et al., 1974).

In summary, all of these results suggest an electrostatic interaction between lysozyme and ovomucin, although it may not be very strong in natural egg white (ionic strength 0.10). The sedimentation equilibrium experiments using native ovomucin enabled reproduction of conditions closer to those of natural egg white than in any earlier studies. Approximately 20–30% of lysozyme in a 1:1 (w/w) native ovomucin–lysozyme mixture was used for the interaction.

It is difficult to estimate the extent of interaction in natural egg white from these data. It is possibly stronger than observed in this study since the protein concentration is higher in natural eggs than was used in these experiments. A dependence of ovomucin–lysozyme aggregation on lysozyme concentration has been reported (Kato et al., 1975; Robinson, 1972). With regard to the mechanism of egg white thinning, the increase in the pH of albumen during storage of shell eggs should weaken the electrostatic ovomucin–lysozyme interaction, if no conformational changes are assumed, due to a decrease in the positive charge of lysozyme as its isoelectric pH is approached. Unfortunately, experiments to investigate the effect of pH on the interaction were unsuccessful because of the tendency of ovomucin and lysozyme to precipitate out of dilute salt solutions above pH 7.4.

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