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Dissociation of Chicken Egg-White Macroglobulin into Subunits in Acid. Hydrodynamic, Spectrophotometric, and Optical Rotatory Measurements*

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ABSTRACT: The high molecular weight globulin from egg white, variously referred to as "line 18," "component 18," and "ovomacroglobulin," has been characterized by physical techniques including sedimentation velocity and equilibrium, diffusion, viscosity, optical rotatory dispersion, and ultraviolet absorption. The native macroglobulin is a glycoprotein of mol wt 6.5×10^5. It appears to have little α-helix content. Spectrophotometric titrations show that most of its phenolic chromophores are abnormal. The transition temperature, T_m, of the native globulin at neutral pH is approximately 60°. In acid solution, the macroglobulin dissociates into two subunits of equal weight which have essentially the same frictional ratio (1.6) as the native molecule. The pH dependence of subunit formation was determined by hydrodynamic and optical methods. The change in ultraviolet absorption parallels the appearance of subunit, but changes in optical rotation do not. The change in the ultraviolet absorption spectrum observed upon separation of subunits and solvent perturbation difference spectra of both the native protein and its subunits indicates that, upon dissociation, 24 phenolic and 2 indole chromophores per subunit become newly exposed to solvent.

The high molecular weight globulin of avian egg white has been called "component 18" or "line 18" because of its position on starch gel electrophoresis (Lush, 1961). Miller and Feeney (1966), who propose the name "ovomacroglobulin," have shown that this protein prepared from the egg white of different avian species exhibits extensive cross-reactivity when tested against antibodies to the chicken protein. The amino acid composition of this globulin is similar to that of the α_2-macroglobulin from human plasma (Dunn and Spiro, 1967), which binds trypsin. The bound trypsin loses most of its proteolytic activity but little of its esterolytic activity (Haverback *et al.*, 1962). We find that the macroglobulin from chicken egg white dissociates into subunits like those formed by the human macroglobulin (Razafimahaleo and Bourrillon, 1968). This paper presents a study of the pH dependence of this dissociation and of the molecular weights and conformations of the subunits.

Experimental Methods[1]

Preparation of Ovomacroglobulin. Fresh egg white (4 l.) was blended and twice dialyzed overnight at 4° against fresh portions of 40 l. of deionized water. The precipitate was centrifuged and discarded. The supernatant was adjusted to

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[1] Reference to a company or product name does not imply approval or recommendation of the product by the U. S. Department of Agriculture to the exclusion of others that may be suitable.

pH 6.5 with acetic acid and brought to 0.30 saturation with saturated $(NH_4)_2SO_4$. After settling overnight at 4°, the precipitate was centrifuged and discarded and the supernatant was brought to 0.45 saturation with saturated $(NH_4)_2SO_4$. The precipitate, after settling overnight at 4°, was collected by centrifuging. The supernatant was discarded.

The precipitate was transferred to a dialysis tube and dialyzed until dissolved. After clarifying in the centrifuge, the solution was applied to a 6.5 × 125 cm column of Bio-Gel P-150 and eluted at 100 ml/hr with 0.10 M KCl. The front peak (*ca.* 0.1% of the original egg-white protein) is primarily "line 18." Front peak fractions were combined, concentrated to about 25 ml by ultrafiltering, and passed through the Bio-Gel column a second time to remove low molecular weight contaminants. The leading 75–80% of the front peak was concentrated to about 50 ml. Saturated $(NH_4)_2SO_4$ was slowly added until a slight precipitate formed (0.3–0.35 saturation). This precipitate was centrifuged off and discarded. The supernatant was then brought to 0.50 saturation with $(NH_4)_2SO_4$ and the precipitate was collected by centrifuging. The purified macroglobulin was dissolved in a minimum amount of water, dialyzed against 0.10 M KCl, and stored at 4°.

Chemical Characterization. Amino acid and carbohydrate analyses were carried out as described previously (Davis *et al.*, 1969). Free sulfhydryl groups were determined by the method of Ellman (1959), both in the presence and absence of denaturing concentrations of guanidine hydrochloride.

Physical Characterization. Two different Beckman-Spinco Model E ultracentrifuges were used for sedimentation velocity experiments. One was equipped with the older speed control and schlieren optics. The other, also used for sedimentation equilibrium, was equipped with electronic speed control, ultraviolet optics, and photoelectric scanner. The "stairsteps" calibration of the photoelectric scanner was checked by measuring the absorption of standard tyrosine solutions against solvent in a double-sector cell under operating conditions, as described by Lamers *et al.* (1963). Diffusion, velocity, and equilibrium experiments were performed near 20°.

Sedimentation velocity was measured from the movement of boundary positions taken as the peak of a schlieren boundary or the position of half-height of an absorption boundary. The positions of schlieren boundaries were measured with the plate-measuring system of Bartulovitch and Ward (1965). For *s vs. c* dependence studies, concentrations reported are the average concentrations for the run. Calculations of sedimentation coefficients and corrections for radial dilution were carried out on an Underwood-Olivetti Programma 101 by use of a moving-boundary *s*–rate program (PI-1016) kindly provided by Dr. Rodes Trautman. Proportions of components present in schlieren pictures were estimated from peak areas.

Sedimentation equilibrium experiments were performed at both high and low speed with approximately 2.5-mm columns. To maintain stability of the gradients formed, sucrose (1.5%) was added to the solutions (Yphantis, 1964). The RTIC unit was used only to measure rotor temperature, not to regulate it. The Evapotrol was adjusted so that the indicated temperature of the rotor was near 20° throughout the run. Both the two-hole aluminum An-D and the four-hole titanium An-F rotor were used. At equilibrium, the distribution pattern of the protein was determined by means of the scanning absorption optical system at a wavelength of 280 mμ. The absorption pattern of the solvent plus protein was measured at the equilibrium speed, and then the rotor was spun at 60,000 rpm to sediment the protein. Then the absorption of the solvent was determined in a separate scan (Schachman and Edelstein, 1966). The protein distribution pattern was taken as the difference between the two curves. Guanidine hydrochloride used as a denaturing solute in physical studies was prepared from the carbonate purified according to Nozaki and Tanford (1967).

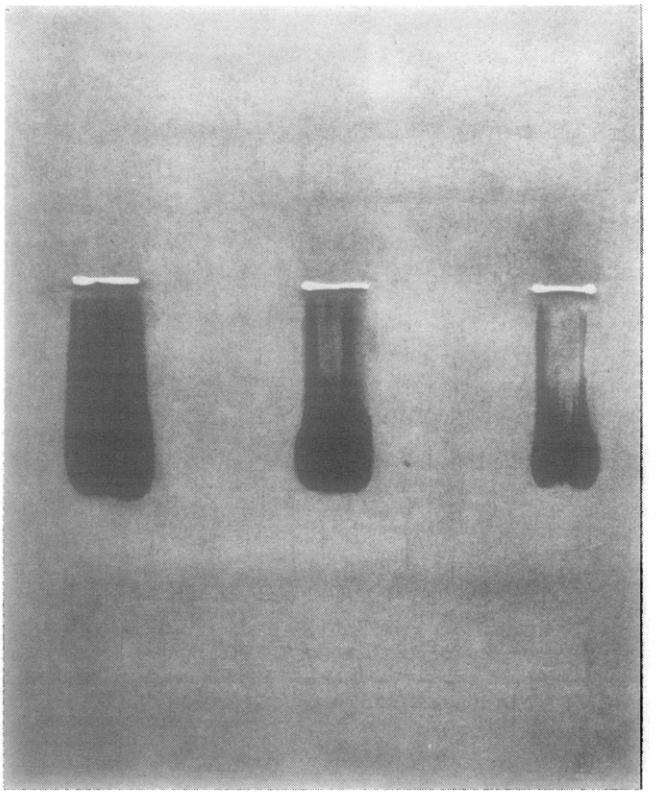

FIGURE 1: Starch gel electrophoresis of purified ovomacroglobulin. Trailing of the anodically moving zones is produced by the high protein concentrations employed in order to show absence of minor contaminants.

Diffusion experiments carried out in the centrifuge were performed in a 12-mm double-sector synthetic boundary cell at speeds near 10,000 and 15,000 rpm. The time of boundary formation was noted and scans of the boundary were obtained at fixed intervals thereafter by means of the photoelectric scanner. The diffusion coefficient was calculated from the Faxén solution to the differential equation for the ultracentrifuge (Schachman, 1959) in the form

$$\left[\left(\frac{\Delta x}{\Delta c}\right)_{\max}\left(\frac{c_0}{Fe^{2\omega^2\bar{s}t}}\right)\right]^2 = D\left[\frac{2\pi(e^{2\omega^2\bar{s}t}-1)}{\omega^2\bar{s}}\right] \qquad (1)$$

Here, $(\Delta x/\Delta c)_{\max}$ has been taken as the maximum slope of the boundary at time t. F is the magnification factor of the optical system, c_0 the concentration measured as the height of the plateau at the beginning of the run, $\bar{s}$ the sedimentation coefficient of the boundary, and $\omega = 2\pi$ rpm/60 sec^{-1}. Equation 1 takes into account radial dilution and variation

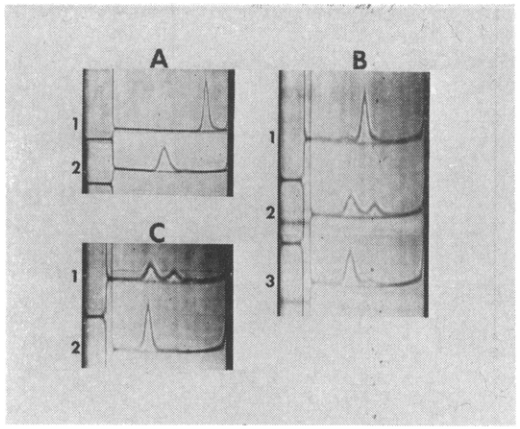

FIGURE 2: Sedimentation of ovomacroglobulin at 20°. (A) Sedimentation of native and subunit at 42,040 rpm: (1) native, 5.7 mg/ml, at pH 9.0 (0.1 M glycine–NaOH) after 70 min, bar angle 70°; (2) subunit, 4.2 mg/ml, at pH 2.5 (0.1 M glycine–HCl) after 69 min, bar angle 65°. (B) Intermediate stages in the dissociation, observed after sedimentation for 25 min at 60,000 rpm, protein concentration 3 mg/ml in 0.1 M NaCl: (1) pH 5.3, bar angle 55°; (2) pH 4.6, bar angle 55°; (3) pH 3.9, bar angle 50°. (C) Reconstitution experiment at 3 mg/ml, in 0.02 M NaCl. Sedimentation for 28 min at 60,000 rpm, 55° bar angle: (1) solution pH adjusted to 3.2 for 1.5 hr at 4°, then neutralized to pH 7; (2) aliquot of pH 3.2 solution, not neutralized.

of the centrifugal field with position in the cell. It does not take into account dependence of s upon c. However, the absorption optical system permits accurate measurement of the boundaries at protein concentrations less than 1 mg/ml. Accordingly, the solution of the ultracentrifugal equation which includes the dependence of s upon c (Fujita, 1956) was not used to calculate the diffusion coefficient. The results obtained appear to justify this approximation. Plots of the data do not show the curvature demonstrated by Baldwin (1957) when self-sharpening is neglected at higher protein concentrations. Essentially the same diffusion coefficients were obtained from height–area measurements of 6-hr free-boundary diffusion runs in a Tiselius apparatus. A Perkin-Elmer Model 38A electrophoresis apparatus thermostated at 20° was used for this purpose. Diffusion coefficients were corrected for temperature, viscosity, and density.

Optical rotation was measured in a water-jacketed 10-cm path-length cell with a Perkin-Elmer Model 141 polarimeter, at the 578-, 546-, 436-, 365-, and 313-mμ mercury lines. The dispersion parameters a_0 and b_0 were calculated from the Moffitt–Yang equation (Urnes and Doty, 1961) with λ_0 assumed equal to 212 mμ. The dispersion of the refractive index of solutions of urea was taken from Warren and Gordon (1966) and Foss *et al.* (1963),[2] of guanidine hydrochloride from Tanford *et al.* (1967), and of water from Chéneveau (1930). Protein concentration was determined spectrophotometrically, using $E^{1\%}_{1\,cm}$ 8.6 (278 mμ), calculated from the dry weight of aliquots of neutral solutions of macroglobulin of known absorption.

Viscosity was measured at 25° with a Cannon-Übbelohde No. 75 dilution viscometer. Intrinsic viscosity was calculated by the method of Tanford (1955).

[2] Quoted by G. D. Fasman.

TABLE I: Amino Acid and Carbohydrate Composition of Chicken Ovomacroglobulin.

Component	Moles/10^5 g[a]
Lysine[b]	44.8 ± 0.6
Histidine[b]	14.3 ± 0.2
Arginine[b]	28.3 ± 0.2
Aspartic acid[b]	81.0 ± 0.5
Threonine[c]	50.5 ± 0.5
Serine[c]	63.0 ± 1.2
Glutamic acid[b]	91.8 ± 0.3
Proline[b]	34.3 ± 0.6
Glycine[b]	39.9 ± 0.3
Alanine[b]	46.1 ± 0.2
Half-cystine[c]	14.0 ± 0.6
Valine[d]	64.7 ± 0.3
Methionine[b]	15.8 ± 0.8
Isoleucine[d]	51.7 ± 1.0
Leucine[b]	71.7 ± 0.3
Tyrosine[c]	30.8 ± 0.3
Phenylalanine[b]	38.1 ± 0.2
Tryptophan[e]	9.3
Glucosamine[c]	31
Ammonia[e]	85.7 ± 7.9
Hexose[f]	0.28 ± 0.03%

[a] Dry weight basis. Kjeldahl N determined on dry protein before hydrolysis: 14.8%. N recovered on column chromatography of hydrolysates: 15.0%. Calculated weight recovery from column: 95.3%. Values reported are from 20- and 70-hr hydrolysates on one preparation, and 20-, 40-, and 70-hr hydrolysates on another. The standard deviation of the mean of the five analyses (or standard deviation of the extrapolated intercept at zero time) is given. [b] Average value of five analyses. [c] Extrapolated to zero time. [d] Value at 70-hr hydrolysis (error estimated). [e] Method of Bencze and Schmid (1957). Tyr/Trp = 3.30. [f] Orcinol method of Schönenberger *et al.* (1957).

Ultraviolet spectra, difference spectra, and perturbation difference spectra were obtained at room temperature (22°) in 1-cm path cells with a Cary Model 15 spectrophotometer. Perturbation difference spectra (Herskovits and Laskowski, 1960, 1962) obtained by addition of ethylene glycol (20%, v/v) were corrected for absorption of ethylene glycol measured separately. Spectrophotometric titrations carried out with the solvent as reference solution were corrected for pH dependence of the solvent absorption. Measurements were made sufficiently long after pH adjustment that they may be considered infinite-time measurements.

Starch gel electrophoresis experiments were carried out by the method of Poulik (1957), as described by Garibaldi *et al.* (1968). The ability of the macroglobulin to inhibit trypsin was tested with hemoglobin, casein, and tosylarginine methyl ester substrates as described by Davis *et al.* (1969).

All data amenable to treatment by least-squares fit to a straight line were so treated. These include sedimentation equilibrium, sedimentation velocity, optical rotatory disper-

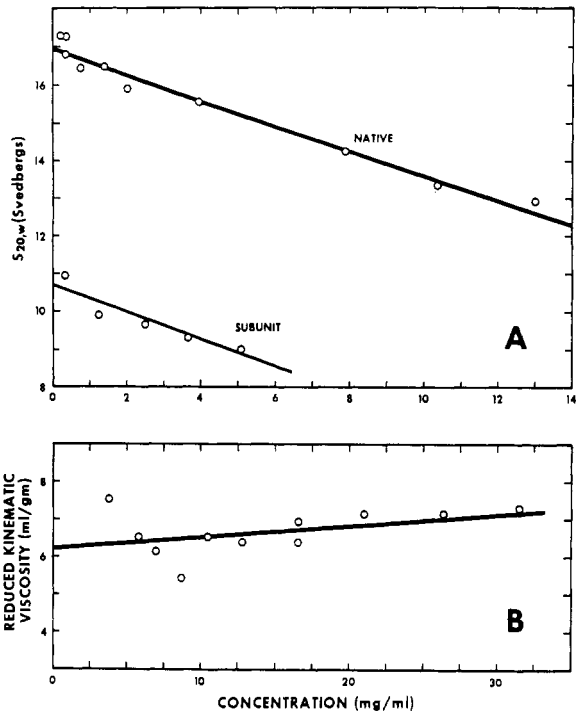

FIGURE 3: Concentration studies. (A) Dependence of the sedimentation coefficient of native and dissociated ovomacroglobulin upon concentration. Conditions: native, in 0.1 M glycine–NaOH (pH 9) and in 0.1 M NaCl (pH 7) at both 44,000 and 60,000 rpm; subunit, in 0.1 M glycine-HCl (pH 2.5) and in 0.1 M NaCl (pH 2.5) at 42,040 and 60,000 rpm. Concentrations are corrected for radial dilution. Sedimentation coefficients determined by a least-squares fit of log $\bar{x}$ *vs.* t were corrected for viscosity, density, and run temperature (21–24°). (B) Concentration dependence of the reduced kinematic viscosity of native macroglobulin in 0.12 M Tris-Cl buffer (pH 8.2) at 25°.

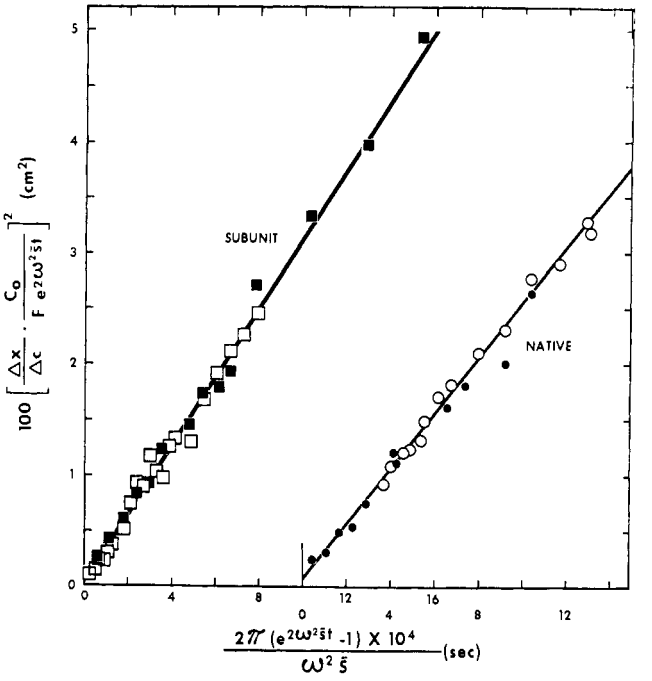

FIGURE 4: Determination of the diffusion coefficient of ovomacroglobulin. The ordinate and abscissa are given in eq 1; the slope of the line is equal to the diffusion coefficient: □, pH 2.5, 0.1 M Tris-glycine, 11,000 rpm, 22°; ■, pH 2.6, 0.1 M glycine-HCl, 15,000 rpm, 22°; ○, pH 9.0, 0.1 M glycine-NaOH, 10,000 rpm, 24°; ●, pH 8.2, 0.1 M Tris-Cl, 15,000 rpm, 22°. Protein concentration was 0.8 to 0.9 mg per ml. Length of run varied from 100 to 200 min.

sion, calculation of diffusion coefficient, intrinsic viscosity, and Hill plots. Limits of error stated are either standard deviations, when the quantity determined is either an intercept or slope of a straight line, or root mean square estimated errors of derived results, calculated as described by Steinbach and King (1950).

Results

The preparation of macroglobulin appears pure by the criterion of starch gel electrophoresis (Figure 1). This preparation does not contain significant amounts of the protein contaminant which moves slightly faster on gel electrophoresis (see Figure 4 of Miller and Feeney, 1966).

The amino acid and carbohydrate analysis of the macroglobulin is given in Table I. Calculated on the basis of dry weight of ovomacroglobulin, Table I indicates a content of the individual amino acids 15% greater, on the average, than that reported by Miller and Feeney (1966), with one exception, our analysis shows 6% less proline. The glucosamine content is slightly greater than the hexosamine content reported by Miller and Feeney. No galactosamine or mannosamine was detected. The hexose content was 0.3%, in contrast to the 3.5–3.8% reported by Miller and Feeney. Although all nitrogen was recovered quantitatively, about 5% of the sample weight is not accounted for by our analysis. All of the half-cystine must be in the form of disulfide, since the determination of free SH groups by the method of Ellman (1959), carried out either in buffer or in the buffered denaturing solvent, 6 M guanidine hydrochloride, shows less than 0.1 free SH group/subunit.

Hydrodynamic Studies. Ovomacroglobulin sediments with a single, nearly symmetrical boundary which travels faster at pH 9 than at pH 2.5 (Figure 2A). Between pH 3 and 5, two peaks are observed in the centrifuge patterns (Figure 2B). The faster sedimenting native form is completely converted into the slower sedimenting form by acidification. All attempts to convert the slower form back into the faster by adjusting pH, temperature, or salt concentration have given incomplete reconstitution. About one-third of the protein was reconstituted at 4° in 0.02 M salt (Figure 2C).

The sedimentation constants of the fast and slow species vary with protein concentration (Figure 3A) according to the equations

$$s_{20,w}\ (\text{native}) = 16.97(1 - 0.020c)$$

$$s_{20,w}\ (\text{subunit}) = 10.72(1 - 0.034c)$$

where c is in milligrams per milliliter and s in Svedbergs. The standard deviations of the slopes of these lines are, respectively, 5 and 20%. Miller and Feeney (1966) report that the $s^0_{20,w}$ of the native macroglobulin is 15.1 S.

Diffusion data obtained in the centrifuge are presented in Figure 4. Free-boundary diffusion of the native macroglobu-

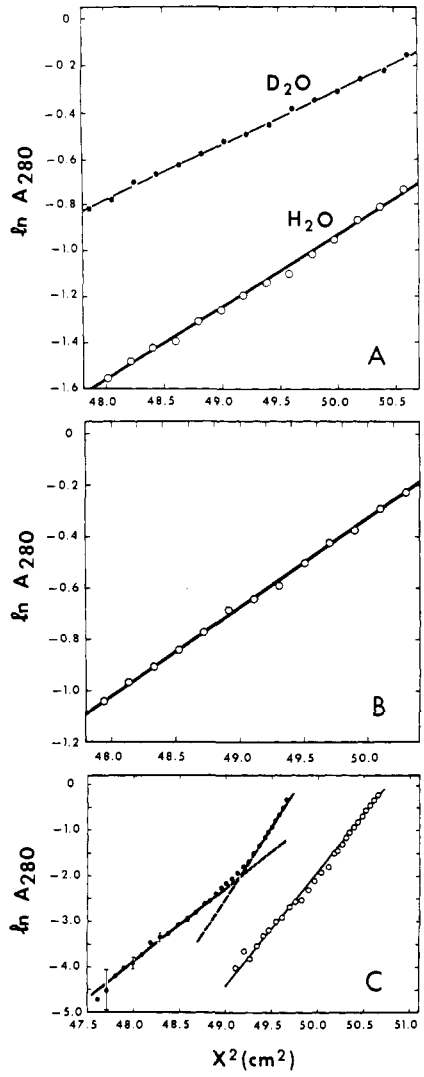

FIGURE 5: Sedimentation equilibrium of ovomacroglobulin. The natural logarithm of the absorption (12-mm path) at 280 mμ (ordinate) is plotted against the square of the distance from the axis of rotation (abscissa). (A) Simultaneous determination of molecular weight and $\bar{v}$ of the native macroglobulin at 22° in 0.1 M glycine–NaOH (pH 9). From start of run, rotor was oversped 3.5 hr at 6000 rpm, held at 2600 rpm for 7 hr, then held at an average rpm of 2993 for 11 hr before these scans were taken. (B) Subunit in D_2O, pH 2.58 in 0.1 M glycine–HCl (1.5% sucrose added) at 25°. Rotor was oversped 3.5 hr at 10,000 rpm, then held at an average speed of 4765 rpm for 20.5 hr before scan was taken. (C) Ovomacroglobulin at 22°, pH 6 in 6 M guanidine hydrochloride after 14.5 hr at 16,035 rpm, O; in 6 M guanidine hydrochloride, 0.002 M dithiothreitol, 24°, pH 8.1, after 13 hr at 28,000 rpm, ●.

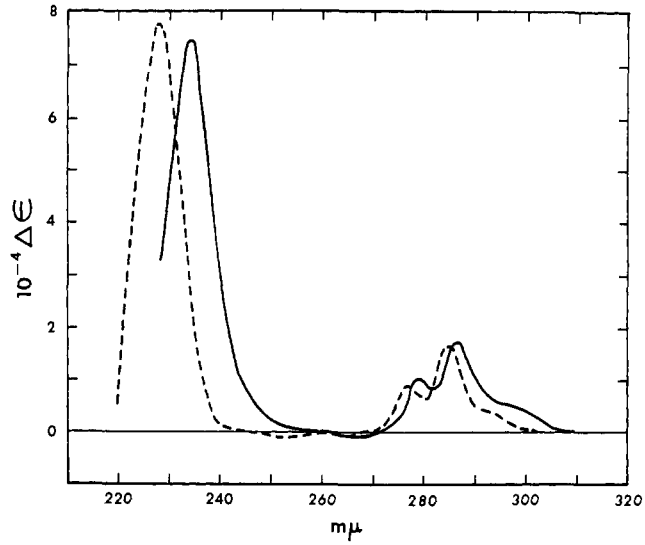

FIGURE 6: Acid difference spectrum of ovomacroglobulin, expressed in terms of molar difference absorption coefficient of the subunit. Solid line: absorption difference observed when a pH 6.5 solution is measured against a pH 2.8 solution as reference. Dashed line: difference spectrum calculated for exposure of 24 phenolic and 2 indole chromophores per subunit.

lin (in 0.01 M phosphate buffer with 0.1 M NaCl present at pH 6.9) and of the dissociated subunits (in 0.01 M glycine-HCl, pH 2.5) was carried out in a Tiselius apparatus at 20°. The diffusion coefficients obtained, listed in Table II, are larger than those reported by Miller and Feeney (1966) by 20% for the native ovomacroglobulin. For calculation of the sedimentation–diffusion molecular weight, the diffusion coefficient at zero protein concentration was assumed identical to the observed value, since for compact molecules, the dependence of D upon c is very small (Wagner and Scheraga, 1956).

The sedimentation diffusion molecular weight of the faster sedimenting form was calculated in the standard way to be $6.59 \pm 0.17 \times 10^5$; that of the slower form, $3.30 \pm 0.09 \times 10^5$. Thus, the native, faster sedimenting form is twice the molecular weight of the slower sedimenting form. The slower sedimenting form has therefore been referred to as the subunit. The molecular weight obtained by us is smaller by 15% than the sedimentation–diffusion molecular weight reported by Miller and Feeney (1966).

The results of sedimentation equilibrium experiments under different solvent conditions are given in Figure 5. The molecular weight of the native protein was found to be $6.37 \pm 0.27 \times 10^5$ (Figure 5A), of the subunit, $3.55 \pm 0.21 \times 10^5$ (Figure 5B), in reasonable agreement with the sedimentation–diffusion molecular weights. At neutral pH, the denaturing solvent 6 M guanidine hydrochloride separates the subunits (Figure 5C), since the molecular weight in this solvent is $3.1 \pm 0.3 \times 10^5$.

When a reducing agent is added to the denaturing solvent, the molecular weight is decreased. For the data shown in Figure 5C, $\bar{M}_w$, the weight-average molecular weight across the cell, is 9.5×10^4, $\bar{M}_m$, the weight-average molecular weight at the meniscus, is 5.4×10^4, and $\bar{M}_b$, the weight-average molecular weight at the cell bottom, is 10.5×10^4. Equilibrium runs at 20,000 rpm give reasonably straight ln c *vs.* x^2 plots, with calculated molecular weights from 9.8 to 11.6×10^4; at 36,000 rpm, $\bar{M}_m$ ranges from 5.6 to 6.8×10^4. Since lower concentrations of dithiothreitol and higher concentrations of mercaptoethanol gave the same results, reduction of the protein appears to be complete.

The partial specific volume of the macroglobulin, determined by sedimentation equilibrium in D_2O and H_2O (Edelstein and Schachman, 1967) and by sedimentation velocity in D_2O and H_2O (Gagen, 1966), and given in Table II was used for calculations of molecular weight. Miller and Feeney

TABLE II: Physical Constants of Ovomacroglobulin.

	Species	
	Native (pH 6–9)	Subunit (pH 2.5)
Molecular weight		
By sedimentation diffusion	$6.59 \pm 0.17 \times 10^5$	$3.30 \pm 0.09 \times 10^5$
By sedimentation equilibrium	$6.37 \pm 0.27 \times 10^5$	$3.55 \pm 0.21 \times 10^5$
Partial specific volume (ml/g)		
By sedimentation equilibrium	0.744 ± 0.009	
By sedimentation velocity	0.744 ± 0.005	
$s^0_{20,w}$ (S)	16.97 ± 0.10	10.72 ± 0.21
$D_{20} \times 10^7$ (cm²/sec)		
Ultracentrifuge data (0.85 mg/ml)	2.43 ± 0.06	3.06 ± 0.06
Free boundary (2.7 mg/ml)	2.34 ± 0.03	3.00 ± 0.06
Intrinsic viscosity, $[\eta]$ (ml/g)	6.51 ± 0.28	
Scheraga–Mandelkern parameter, β	$2.13 \pm 0.08 \times 10^6$	
Frictional ratio, f/f_{min}	1.56 ± 0.04	1.58 ± 0.04

(1966) report $\bar{v}$ to be 0.745 ml/g. A $\bar{v}$ of 0.728 ml/g was calculated from the amino acid composition (Table I) using the method of Cohn and Edsall (1943) and the tables of partial specific volumes of amino acids and carbohydrates given by Schachman (1957) and Gibbons (1966). For this calculation, the discrepancy between the total weight and weight recovered on analysis was assumed to be hexose ($\bar{v}$ = 0.61 ml/g) since good recovery of nitrogen was obtained. A value of 0.72 ml/g was assumed for the $\bar{v}$ of the macroglobulin in 6 M guanidine hydrochloride, in accordance with the decrease in $\bar{v}$ observed for other proteins in this solvent (Hade and Tanford, 1967).

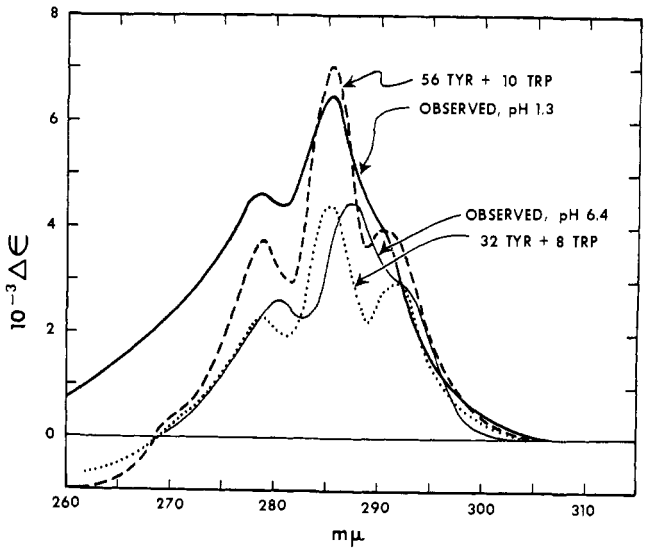

FIGURE 7: Perturbation difference spectra of ovomacroglobulin produced by 20% (v/v) ethylene glycol. The observed perturbation difference spectrum of the native ovomacroglobulin at pH 6.4 is compared with a perturbation difference spectrum calculated for 32 phenolic and 8 indole chromophores exposed per subunit. The observed difference spectrum of the separated subunits at pH 1.3 is compared with a difference spectrum calculated for 56 phenolic and 10 indole chromophores exposed per subunit.

Ultraviolet Absorption Measurements. The acid difference spectrum of ovomacroglobulin (native *vs.* subunit as reference) is shown in Figure 6. The observed difference spectrum is compared with a difference spectrum calculated as described elsewhere (Donovan, 1969) for the exposure of 24 phenolic and 2 indole chromophores per subunit. Perturbation difference spectra of both the native and dissociated macroglobulin produced by 20% (v/v) ethylene glycol are given in Figure 7. The observed perturbation difference spectra are compared with spectra obtained by summation of the perturbation difference spectra of appropriate amounts of tyrosine and tryptophan (Donovan, 1964; Herskovits and Sorensen, 1968).

Studies of the Dissociation in Acid. The pH dependence of the dissociation of the native form was examined using sedimentation velocity, optical rotation, and ultraviolet absorp-

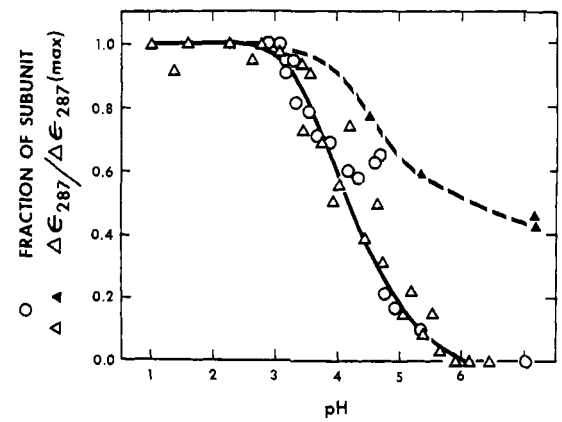

FIGURE 8: The pH dependence of the dissociation of ovomacroglobulin: O, the fraction of subunit determined from area measurements of schlieren pictures; Δ, relative change in absorption at 287 mμ, *vs.* reference solution pH 7; ▲, attempted reversal (from pH 3) of the dissociation, measured by absorption change at 287 mμ. Absorption data were obtained in 0.1 M Tris-glycine, in 0.1 and 0.5 M KCl. Since there was no apparent difference due to solvent, data points for these three solvents are not distinguished.

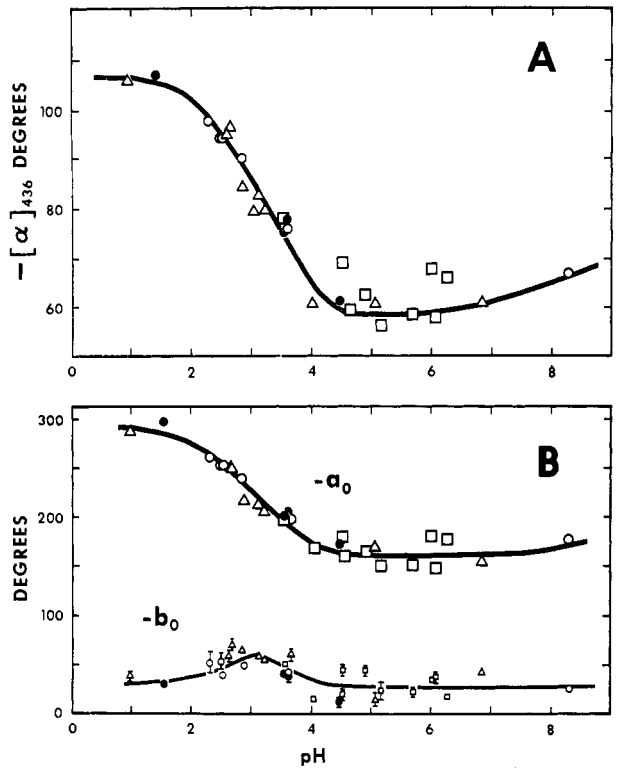

FIGURE 9: pH dependence of the optical rotation of ovomacroglobulin at 22°. (A) The levorotation measured at the 436-mμ mercury line. (B) The optical rotatory dispersion parameters a_0 and b_0. O, 0.1 M Tris-glycine; △, water (no added salt); ●, 0.1 M KCl or NaCl; □, 0.5 M KCl. For the b_0 measurements, the bar length is two standard deviations; when no bar is shown, its length was equal to, or less than, the size of the symbol.

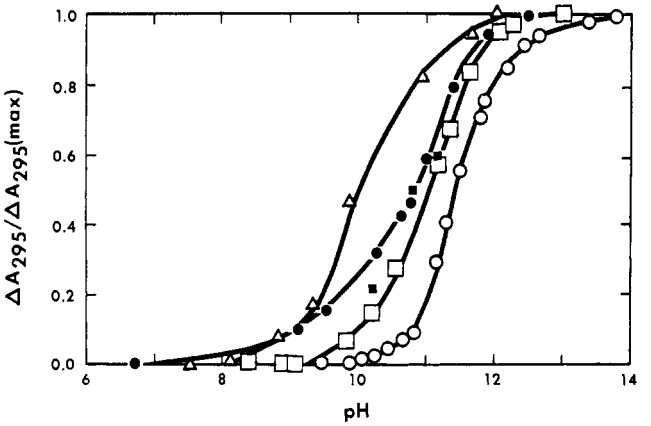

FIGURE 10: Titration of the phenolic groups of ovomacroglobulin at 22°. The fraction of phenolic groups ionized (ordinate) is plotted against pH (abscissa): O, forward (to higher pH) titration in water, and ●, reversal; □, forward titration in 8 M urea, and ■, reversal; △, forward titration in 6 M guanidine hydrochloride.

tion. The fraction of subunit is shown as a function of pH in Figure 8. Near pH 4.5, the isolectric point of the protein (Miller and Feeney, 1966), the relative area obtained for the slowly sedimenting peak is unreliable, since it is evident from the pictures that the native protein aggregates to form faster sedimenting species, reducing the area under the faster sedimenting peak. The proportion of subunit present is thus overestimated. Corrections for this aggregation were not made, since failure of the schlieren patterns of Figure 2B to coincide with the solvent base line between the two peaks is evidence for a possible continuous equilibration between native and dissociated species during centrifugation. Concentration dependence of the association and Johnston–Ogston effects also introduce errors in estimating the fractions of the two forms present. In addition, schlieren pictures taken at longer times in runs near pH 4.8 showed a broad peak moving with intermediate velocity. Such an intermediate peak can appear in a monomer–dimer system under transport when the rates of the association and dissociation reactions are comparable with the transport velocity (Cann and Bailey, 1961; Belford and Belford, 1962).

The change in ultraviolet absorption (Figure 8) correlates well with amount of the slowly sedimenting component measured from the areas of schlieren peaks. As with sedimentation velocity, the change in absorption is not completely reversed when the pH of an acid solution of ovomacroglobulin is increased (Figures 2 and 8). Optical rotation (Figure 9) does not have the same pH dependence as ultraviolet absorption or dissociation into subunits. Although the dissociation is half complete at pH 4.2, half of the optical rotation change as measured by $[\alpha]_{436}$ (Figure 9A) is completed only at pH 3.1. The change in a_0 and b_0 with pH is shown in Figure 9B. When acid solutions of ovomacroglobulin were adjusted to neutrality, very little reversal of the change in optical rotation occurred.

Stability of the Ovomacroglobulin at Alkaline pH. Titration of the phenolic groups in native ovomacroglobulin is markedly abnormal (Figure 10). Above pH 11, the titration curve is very steep and irreversible, suggesting denaturation produced by electrostatic forces. In 8 M urea, the titration curve of the phenolic groups is shifted about 0.4 pH unit to lower pH. In 6 M guanidine hydrochloride the pK' of the phenolic groups is 10.0, a value characteristic of normal phenolic groups. In 6 M guanidine hydrochloride, phenol has a pK' of 10.2 (Donovan *et al.*, 1959) and the phenolic group in model compounds has a pK' of 10.0 (Edelhoch, 1967). By this criterion, 6 M guanidine hydrochloride completely denatures ovomacroglobulin, but 8 M urea does not.

At pH 11.9, ovomacroglobulin sediments as a single boundary with $s_{20,w}$ of 9.9 S (at 0.5 mg/ml). Since 80% of the phenolic groups are ionized at this pH, the 9.9S species is probably a subunit with altered conformation. We have not attempted to characterize this globulin at high pH.

Effects of Denaturants at Neutral pH. Guanidine hydrochloride separates the subunits (Figure 5C). The optical rotatory parameters for the macroglobulin in 6 M guanidine hydrochloride at pH 6 are: $a_0 = -522°$, $b_0 = 104°$, and $[\alpha]_{436} = -185°$. This a_0 value agrees with those observed for other proteins denatured in 6 M guanidine hydrochloride (Tanford *et al.*, 1967), but the b_0 value does not. Urea at concentrations up to 3 M does not affect the optical rotation; above 3 M, a_0 increases roughly linearly with urea concentration. At a concentration of 9 M urea, $a_0 = -480°$, $b_0 = 20°$, and $[\alpha]_{436} = -178°$.

Heat Stability. The temperature dependence of the optical rotation of ovomacroglobulin is shown in Figure 11. The transition temperature of the native protein appears to be

TABLE III: Comparison of the pH Dependence of Appearance of Subunit, Change in Ultraviolet Absorption, and Change in Optical Rotation.

Measurement	Hill Plot Parameters	
	Characteristic pH ± Std Dev	Slope ± Std Dev
Fraction of subunit	4.20 ± 0.20	−0.96 ± 0.06
Ultraviolet absorption change (ΔA_{286})	4.30 ± 0.29	−0.94 ± 0.06
Optical rotation change ($\Delta[\alpha]_{436}$)	3.08 ± 0.17	−0.97 ± 0.04

62–64°. The upper parts of the melting transition (Figure 11) could not be determined, because of formation of turbidity upon heating, so an extrapolation of the optical rotation data at lower pH was used to estimate the final value at high temperature for the experiments at pH 6.1 and 8.3. At all values of pH, the parameter b_0 was determined to be −25 ± 10° throughout the melting range (above 30°). Thus, no melting transition is revealed by this parameter. The parameter a_0 is linearly related to $[\alpha]$, as expected when no change in b_0 occurs. The highest value of a_0 observed in the melting experiments (Figure 11) is −350° at pH 3.6 and 70°.

Proteolytic Inhibition Experiments. The ovomacroglobulin produced no significant inhibition of trypsin activity upon protein and ester substrates, even at high ratios of globulin to trypsin. Addition of macroglobulin did not affect the inhibition of trypsin by subsequently added soybean trypsin inhibitor. Binding experiments carried out in the ultracentrifuge indicate that neither trypsin nor chymotrypsin are bound by the macroglobulin when present in solution in approximately equimolar amounts.

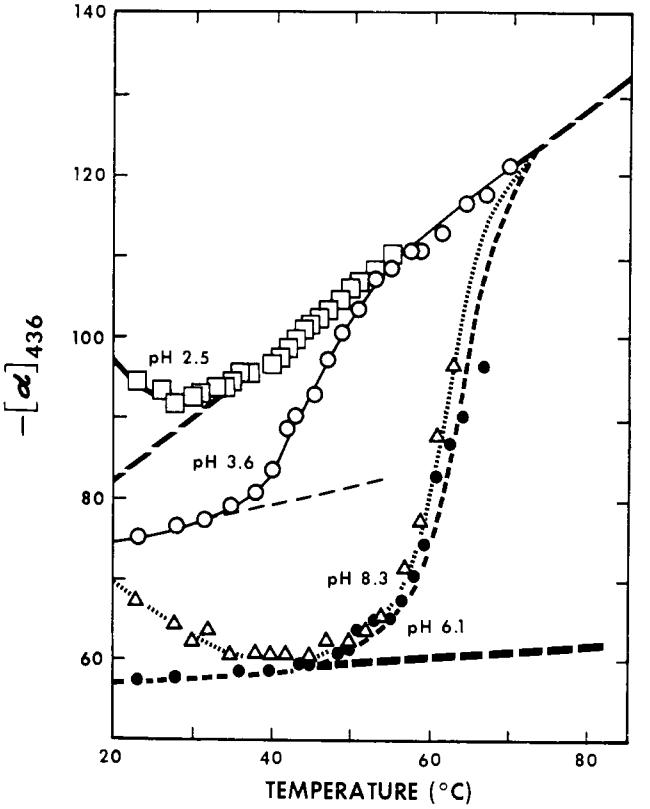

FIGURE 11: Temperature dependence of the optical rotation of ovomacroglobulin at 435.8 mμ. Solvents: △, water; ●, 0.5 M KCl; ○ and □, 0.1 M Tris-glycine–HCl.

Discussion

The amino acid composition of ovomacroglobulin (Miller and Feeney, 1966, and Table I) is similar to that reported for human α_2-macroglobulin by Dunn and Spiro (1967). The correspondence of composition and molecular weight suggests that ovomacroglobulin is analogous to serum α_2-macroglobulin. Gel electrophoretic patterns of chicken egg white, chicken serum, and human serum are similar with respect to the positions of the corresponding albumins, transferrins, and macroglobulins (Garibaldi *et al.*, 1968; Allison and Feeney, 1968; Dunn and Spiro, 1967; Mehl *et al.*, 1964).

Using the physical constants from Table II, the Scheraga–Mandelkern (1953) parameter β was found to be 2.15 ± 0.06 × 10^6 (sedimentation diffusion molecular weight used) or 2.11 ± 0.08 × 10^6 (sedimentation equilibrium molecular weight used). The ovomacroglobulin is probably roughly spherical. Its effective hydrodynamic volume, V_e, was calculated as indicated by Scheraga and Mandelkern (1953). A theoretical minimum volume, V_{min}, was obtained by multiplying the molecular weight by the partial specific volume. The ratio V_e/V_{min} is 3.6 or 2.4, assuming limiting axial ratios of 1 or 3, respectively. This suggests that a large amount of water is hydrodynamically associated with the macroglobulin. Calculated frictional ratios are given in Table II. Here, f_{min} is the frictional coefficient calculated (Tanford, 1961) for an equivalent unhydrated sphere. The change in frictional coefficient expected upon association of two roughly spherical subunits is within the limits of error in the determination of the frictional coefficient.

The hydrodynamic measurements indicate that no *gross* changes in size, shape or in amount of hydrodynamically associated water occur when the subunits separate, since the ratio: $s^{3/2}$ (native)/$s^{3/2}$ (subunit) is 1.99, and the ratio of the diffusion coefficients of the subunit and native protein is 1.27 ± 0.01. Since addition of urea or guanidine hydrochloride also produces dissociation, the binding interaction between the subunits cannot be a covalent one.

There are at least two steps in the conversion of the native macroglobulin into the separated subunits at pH 2. A comparison of Figures 8 and 9 shows that the changes in ultraviolet absorption coincide with the appearance of the slower sedimenting subunit, but that the changes in optical rotation are displaced about one pH unit toward lower pH. This is shown more clearly by a comparison of plots of log $[f/(1 - f)]$ *vs.* pH (Hill plots), where f is the fraction of slowly sedimenting subunit, or ΔA_{286}, or $\Delta[\alpha]_{436}$. Least-squares fits of the data of Figures 8 and 9 to a linear relation between log $[f/(1 - f)]$ and pH give the characteristic pH values (the pH at which $f = 0.5$) and slopes shown in Table III. Within the precision of the experimental data, the fraction of subunit formed shows the same pH dependence as the change in ultraviolet

absorption. Because the slopes listed in Table III are close to unity, the interactions of *titratable* groups of the macroglobulin which stabilize both the association of subunits and their conformation appear noncooperative.

When the macroglobulin dissociates in acid solution, only a small decrease in absorption occurs ($\Delta\epsilon_{286}/\epsilon_{278} \simeq 0.04$). Solvent perturbation of the native and dissociated macroglobulin (Figure 7) indicates that upon dissociation, the fraction of phenolic chromophores exposed to solvent increases from 0.30 to 0.53 and the fraction of the indole chromophores exposed increases from 0.25 to 0.31. The solvent perturbation difference spectra agree with the acid difference spectrum (Figure 6) in regard to the number of chromophores newly exposed to solvent. Despite the apparently large amount of water hydrodynamically associated with the ovomacroglobulin, considerable portions of the subunit structure are not in contact with the solvent water.

The transition temperature of the native globulin is close to 60°, the temperature used for pasteurizing egg white (Lineweaver *et al.*, 1965). This agrees with the loss of macroglobulin shown by starch gel electrophoresis of pasteurized egg white and whole egg (J. J. Clary, unpublished results). If a convenient quantitative assay were available, determination of the amount of macroglobulin present in egg white or whole egg might be used to determine whether these products had been pasteurized.

The absence of any inhibitory effect of the macroglobulin on trypsin activity, apart from showing the absence of ovomucoid and ovoinhibitor contaminants, suggests that the macroglobulin is not fully analogous to the human serum α_2-macroglobulin. However, Mehl *et al.* (1964) observed that the trypsin-binding property of the serum protein is destroyed by exposure to ammonium ion concentrations of 0.2 M or greater. Since our preparations were fractionated with ammonium sulfate, the possible loss of the inhibiting property during isolation merits further investigation.

Acknowledgments

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The Identification of Aspartic Acid Residue 52 As Being Critical to Lysozyme Activity*

S. M. Parsons† and M. A. Raftery

ABSTRACT: A single ethyl ester derivative of lysozyme which retains inhibitor binding capability but which is catalytically inactive has been identified as occurring at aspartic acid residue 52 in the amino acid sequence.

Recent discussions of hen egg-white lysozyme generally have attributed its glycosidic activity to the side chains of Glu-35 and Asp-52. There is some kinetic evidence that carboxyls participate (Osawa, 1966; Raftery and Rand-Meir, 1968; Rupley and Gates, 1967). Also, modification of all "available" carboxyls inactivates the enzyme (Fraenkel-Conrat, 1950; Hoare and Koshland, 1967; Parsons *et al.*, 1969). However, the evidence that Glu-35 and Asp-52 are the specific carboxyls which are involved has been of a presumptive nature, based on the elegant results of Philips, Blake, and coworkers for the crystalline enzyme and on their model building (Blake *et al.*, 1967a,b, 1965; Blake, 1966).

We previously had utilized triethyloxonium fluoroborate to achieve mild selective esterification of carboxylates in lysozyme. We were able to isolate and partially characterize two single ethyl ester derivatives of the enzyme. One of these is enzymatically inactive but still retains the capability of binding the competitive inhibitor chitotriose. The details regarding the preparation and some of the properties of this inactive ester are given by Parsons *et al.* (1969). Figure 1 illustrates the chromatographic pattern obtained in the separation of a mixture of the lysozyme ester derivatives. Component II is the enzymatically inactive single ester discussed here. This communication describes the identification of this important carboxylic acid side chain.

Experimental Section

Materials. Hen egg-white lysozyme (lot 77B-8040) was purchased from Sigma Chemical Co. Bovine α-chymotrypsin (lot T-97207) was obtained from Armour Research Division, aminopeptidase M (lot 51132, 12,500 mEU/mg) from Rohm and Haas, subtilisin Carlsberg (lot 50624) from Novo Industries, Copenhagen, and CPA[1] (lot 762) from Worthington Biochemical Corp. Ninhydrin was a product of the Pierce Chemical Co. and aspartic acid was obtained from Eastman Kodak. Poly-L-glutamic acid was a product of Schwarz BioResearch, Inc. Standardization buffers for the pH meter were from Beckman.

The β-ethyl ester of aspartic acid was synthesized by refluxing a mixture of 5.0 g of DL-Asp-HCl and 1.1 g of anhydrous HCl in 50 ml of absolute ethanol for 15 min. The resulting warm solution was brought to cloudiness with dry ether and set aside (Bergmann and Zervas, 1933). Crystalline β-DL-ethylaspartic acid hydrochloride (2.8 g, mp 174.5–177.5°) was obtained. The structure was confirmed by nuclear magnetic resonance spectroscopy.

The γ-ethyl ester of glutamic acid was quickly synthesized in microamount by esterification of 1 mg of poly-L-glutamic acid with triethyloxonium fluoroborate in a manner similar to the lysozyme esterification. The esterified polymer was treated with subtilisin and aminopeptidase M as described below, yielding a solution containing essentially only L-glutamic acid and γ-ethyl-L-glutamate in about 1:3 ratio.

Analytical Methods

General Procedures. Acid hydrolysis of peptides was effected in constant-boiling HCl under vacuum for 20 hr at 105°. Base hydrolysis of protein samples was effected in 4 N barium hydroxide by the method of Noltman *et al.* (1962). Amino acid analyses were performed on a Beckman-Spinco Model 120B amino acid analyzer. The sodium buffer system usually employed was similar to that of Spackman (1963). Asparagine and β-ethylaspartic acid (β-EtAsp) were completely resolved from the other amino acids at 25° in a

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[1] Abbreviation used is: CPA, carboxypeptidase A.