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The Buoyant Titration of Native and Carbamylated Bovine Serum Mercaptalbumin†

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ABSTRACT: The buoyant density titration curves of native and carbamylated bovine serum mercaptalbumin were measured throughout the pH range 5.3–12.7. Large increments in the buoyant density were observed above pH 10, with inflection pH values of 11.2 and 11.4 for native and carbamylated bovine serum mercaptalbumin, respectively. For the modified protein in which 25 out of 58 lysine residues were carbamylated, the buoyant densities were 0.048 g/ml higher at neutral pH and 0.024 g/ml higher at the extrapolated pH 13. The carbamyl groups apparently produce a larger residual density at pH 13 than they did in the case of ovalbumin. Homopolymer buoyant density titration data were demonstrated to be of value in calculating the contributions of titratable residues to the buoyant density of both proteins. The buoyant density increment at high pH was due largely to the deprotonation of the lysines as indicated by the diminished change in buoyant density between pH 10 and 12.7 for the modified protein. These density changes were attributable primarily to a gain of cesium ions. The limited modification of the lysine residues under mild reaction conditions and the rather high intrinsic dissociation constant of tyrosine residues in mercaptalbumin may indicate a preferential modification of easily accessible lysine residues. Phenolic deprotonation is facilitated by the neutralization of normally charged lysine residues and demonstrates ionic interactions between internal lysines and certain carboxyl and tyrosine residues thereby stabilizing the native state of the protein.

The use of well-known amino acid blocking groups has found widespread employment as a means of studying various biophysical properties of proteins when specific ionizable residues are chemically modified. The technique of the carbamylation of lysine residues (Stark and Smyth, 1963) (Svendsen, 1967) was adopted for the modification of native bovine serum mercaptalbumin. The buoyant behavior of native and carbamylated BMA[1] was studied in the pH range 5.3–12.7. This work follows the recent study of the buoyant density titration of native and carbamylated ovalbumin (Ifft, 1971), the only other protein for which such data have been obtained.

BMA is a widely studied protein, characterized by its exceptional ability to bind anions and water. Although BMA

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[1] Abbreviation used is: BMA, bovine serum mercaptalbumin.

is a very flexible protein, it is not known whether serious conformational changes occur between pH 5 and pH 12.5. Considerable information about the anion binding property of BMA was elucidated by Scatchard et al. (1959) and, more recently, by Williams and Ifft (1969). It was of interest, therefore, to identify the amino acid side chains that participate in this binding, as well as their contribution to the buoyant density of the protein. Of significant importance to the ion binding behavior of BMA are the 58 lysine residues, bearing ionizable ϵ-NH_3^+ groups, which were readily modified with potassium cyanate to a neutral homocitrulline residue as follows:

$$R\text{-}(CH_2)_4\text{-}NH_3^+ \xrightarrow[pH\ 9.0]{OCN^-} R\text{-}(CH_2)_4\text{-}NHC(=O)\text{-}NH_2$$

Density gradient equilibrium ultracentrifugation (Meselson et al., 1957) in a CsCl gradient was used to determine buoyant densities, ρ_0, of the native and carbamylated protein at various pH values. The work of Williams and Ifft (1969) produced the first buoyant density titration curve of a protein, namely BMA. The data obtained were interpreted in terms of Cs^+ and Cl^- binding throughout the pH range 5.0–12.5. Since the buoyant density of the protein is a function of bound ions and degree of hydration, as well as its intrinsic density, any change in these bound species due to changes in pH must be accompanied by changes in ρ_0. Similarly, any modification of amino acid residues will also affect the ions and water that these residues bind, and, hence, this will also change the buoyant density. Therefore, we were interested in demonstrating the following: (1) the differences in the buoyant behavior of native and carbamylated BMA; (2) the changes in ion and water binding of lysine residues due to neutralization of the normally charged ϵ-NH_3^+ groups; (3) the contribution of lysines to the buoyant density of BMA at high pH; (4) the correlation between calculated buoyant density contributions of certain residues (from homopolymer data) and observed contributions obtained from the buoyant density titration curves; and (5) the contribution of the carbamyl groups to the ρ_0 of BMA, and whether this residual density of the modified protein is due solely to these groups.

Materials and Methods

A sample of twice crystallized BMA, purchased from the Nutritional Biochemical Co., lot 8915, was found to be present in both monomer and mercury-dimer forms upon sedimentation velocity analysis. The BMA was purified according to the procedure of Dintzis (1964), by allowing a 10% (w/v) solution of stock BMA, made 2×10^{-3} *M* in sodium bicarbonate and adjusted to pH 6.0, to elute on a column packed with ion exchange resin beds of ammonium, thioglycolate, acetate, mixed OH^- and H^+, and H^+. The cation exchanger used was Dowex 50W-X8, H^+ form, and the anion exchanger was Amberlite IRA-400, Cl^- form. Conversion to the above forms was effected by methods similar to those of Dintzis. Approximately 11 mg of $NaHCO_3$/g of protein was used to obtain the desired pH prior to elution.

A glass column, 30 × 1.5 cm diameter, was employed for purification of 100 ml of 10% BMA. The capacity of each bed was 50–100 times in excess of the stoichiometric requirement. The thioglycolate resin bed darkened considerably as purification of the BMA proceeded, indicating the removal of the bound mercury. The eluent was collected in 5-ml fractions, whose concentrations were determined using a Cary 14 spectrophotometer, and $\epsilon_{1cm}^{280} = 43{,}600$ (Wetlaufer, 1962), mol wt 67,000 (Putnam, 1965). Tubes containing the monomeric protein were pooled, and their final concentration was found to be 7.9% with an isoionic pH of 5.10.

The methods developed by Stark and Smyth (1963) and Svendsen (1967) were adapted for the carbamylation of native BMA. The former requires the use of 8*M* urea in the modification reaction, whereas the procedure outlined by Svendsen employs milder conditions, which have proven successful in the modification of subtilisin, and ovalbumin (Ifft, 1971). The use of high temperature and extreme pH, as well as known protein denaturing agents, were avoided so as to maintain the integrity of the protein.

A Radiometer TTT2 titrimeter and pH-Stat assembly equipped with a constant temperature bath operating at 35° was employed to maintain the pH at 9.0. Reagent grade KOCN was obtained from the Baker Chemical Co.

Approximately 1.25 g of KOCN was added to 8 ml of 5% purified BMA (pH 9.0). The solution was allowed to equilibrate to the proper temperature, whereupon any pH adjustments were effected with 1 *N* NaOH, and 3 *N* HCl, which was employed to maintain the pH at 9.0. This is necessitated due to the hydrolysis of cyanate ion, producing OH^-. The reaction was allowed to proceed for 18 hr at 35°.

Upon completion of carbamylation, the reaction mixture was placed on a Sephadex G-25 column (25 × 2 cm) equilibrated with a 10^{-3} *M* phosphate buffer (pH 7.8). The eluent was collected in fractions of 5 ml each and their concentration was determined spectrophotometrically at 280 nm. Tubes 4 and 5 were found to have a combined protein concentration of 0.8%. This solution was used as the stock solution for all subsequent experiments with the carbamylated protein.

Sedimentation coefficients for both native and carbamylated BMA were measured by standard procedures (Ifft and Vinograd, 1962) in a Spinco Model E analytical ultracentrifuge at 59,780 rpm. A 1.0% solution of stock BMA was prepared in 0.1 *M* acetate buffer (pH 5.15). Photographic analysis indicated the presence of two sedimenting peaks. Upon purification of the stock solution, a 1.0% BMA solution was prepared in acetate buffer, 0.1 *M*, and pH 5.19. Sedimentation analysis demonstrated only a single, nearly symmetrical sedimenting peak, indicating a uniform, homogeneous protein sample. A final run was made after carbamylation of the BMA. The sample of modified mercaptalbumin was 0.8% in 0.1 *M* acetate buffer (pH 5.22).

The number of lysine residues converted to homocitrulline residues was measured by amino acid analyses of the native and carbamylated BMA with the Beckman Model 118 amino acid analyzer.

Three samples of the native and the carbamylated protein were hydrolyzed in 6 *N* HCl for 24, 48, and 72 hr.[2] This analysis enabled an extrapolation back to zero hours hydrolysis time to correct for the gradual destruction of serine, threonine, and tyrosine residues. This was also necessary for the calculation of the homocitrulline residues which are slowly converted to lysines with increasing time of hydrolysis in carbamylated BMA.

For separation, standard methods for amino acid analysis were employed (Moore et al., 1958). This allowed for the homocitrulline to elute just prior to the valine peak on the

[2] Only 24- and 72-hr hydrolysis determinations were made for carbamylated BMA.

chromatogram. The extent of modification was calculated by the difference in lysine content between native BMA and the carbamylated protein.

Density gradient equilibrium analysis of native and carbamylated BMA was performed in cesium chloride gradients using standard methods and techniques (Ifft, 1973).

A Spinco Model E analytical ultracentrifuge equipped with an RTIC unit and employing schlieren optics was used in all experiments. Spinco An-D and An-F rotors were utilized for runs at 59,780 rpm and 52,640 rpm, respectively. Photographic analysis of the schlieren patterns was made using Kodak metallographic plates. Single-sector Kel-F centerpieces, 12 mm, with quartz windows, wedge angles +1 to −2°, were used in runs. The average duration of each run was 24 hr.

A Bausch and Lomb refractometer equilibrated at 25.0° with a constant temperature bath was used to measure $n^{25}D$ values. The solution density, ρ_{25}, was calculated from the quadratic formula for CsCl solutions (Ifft et al., 1970). Refractive index measurements were made before and after each run, as were pH measurements. When a drift in either was observed between the initial and final determinations, the latter value was used. Most solutions had a ±0.0002 or zero drift in $n^{25}D$ and were corrected for protein, 0.1%, by subtracting 0.0002 from the final $n^{25}D$ measured. The pH was measured with a Beckman Research pH meter, Model 1019.

Buffers used in the experiments were selected largely on the basis of their volume change upon ionization, ΔV, near their pK_a values (Weber, 1930). In general, buffering systems exhibiting large ΔV were avoided due to considerable pH shifts effected by the high pressures in the solution column during centrifugation. The following buffers were employed for the pH ranges specified: acetate, pH 5.3–6.0; phosphate, pH 6.8–7.6; carbonate, pH 8.6–10.8; phosphate, pH 11.2–11.5; CsOH, pH 12.6–12.7; borate, pH 9.3 and 12.7. Buffer concentrations were generally 0.02 *M* so that their effects on the density gradient of CsCl or the binding properties of the protein were negligible. Runs made below pH 5.5 were characterized by the appearance of a narrow, aggregated band in the centrifuge cell. This precipitation, caused by the proximity of the pH to the isoelectric point of BMA, did not interfere with the $n^{25}D$ or pH measurements. A temperature of 25.0 ± 0.1° was maintained in all centrifuge runs.

A four-cell run was attempted using the An-F rotor at ω = 52,640 rpm. Solutions of both the native and carbamylated proteins were used. Figure 1 displays the results of this run that was achieved by employing +1°, 0°, −1°, and −2° quartz windows with a single sector Kel-F centerpiece. The bar angle was adjusted to photograph the bottom two bands in Figure 1 for a more precise determination of the band center.

A Fortran IV computer program facilitated the computation of the buoyant densities, ρ_0^0 and ρ_0.

Results

The $s_{20,w}$ value for carbamylated BMA, 4.21 S, compares favorably with $s_{20,w}$ = 4.19 S for the native mercaptalbumin. The initial run yielded values of 6.91 S and 4.32 S for the BMA–Hg dimer and the native, monomer protein, respectively. Williams and Ifft (1969) obtained $s_{20,w}$ values of 6.5 S and 4.25 S for each respective protein. Upon purifying the stock solution in a similar manner, they obtained a $s_{20,w}$ of 4.15 S for the native mercaptalbumin.

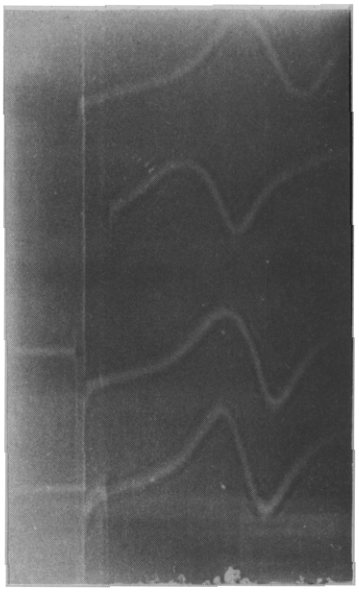

FIGURE 1: Four cell buoyant density analysis of BMA in 2.5 *M* CsCl. Schlieren patterns of the equilibrated bands are from top to bottom: native BMA, pH 10.33 (+1°); carb. BMA, pH 10.36 (0°); native BMA, pH 5.71 (−1°); native BMA, pH 12.66 (−2°).

The relationship, derived from density gradient centrifugation equations (Avruch et al., 1969)

$$\frac{(M_{carb})^{2/3}}{s_{20,w}} \simeq \frac{(M_{nat})^{2/3}}{s_{20,w}}$$

was employed to ascertain the correlation between the two proteins in terms of approximate molecular weights and sedimentation properties. According to the parameters for native BMA (M_{nat} = 67,000, $s_{20,w}$ = 4.19 S), the $s_{20,w}$ for the modified protein, M_{carb} = 68,050, is calculated to be about 4.23 S, within 0.5% of the experimentally determined value. Because the expected error in each $s_{20,w}$ is about 1%, the agreement is rather good.

The measured amino acid compositions are given in Table I, along with several amino acid compositions from the literature. The 62 lysine residues reported by Spahr and Edsall (1964) are in contrast to the lower values obtained by Stein and Moore (1949), 58 lysines, and by Brand (1946), 56 lysines. More recently, King and Spencer (1970) have reported 57–58 lysines, and, in this work, a value of 58 lysine residues was obtained for the native protein.

Figure 2 demonstrates the calculated values of lysine and homocitrulline residues as a function of hydrolysis time. Production of lysine residues, due to the conversion of homocitrullines to lysines, with increased time of hydrolysis reaches a value of 55 after 72 hr of hydrolysis for the modified protein. This leaves approximately 3 homocitrulline residues. The extrapolated values at zero time are 33 lysines and 25 homocitrullines, or a 43% modification by treatment with OCN^-. These values are estimated to have an accuracy of about ±1 residue. The conversion of only 25 of the 58 lysine residues may indicate that at least some of the lysines lie in relatively less accessible sites, thus making them less susceptible to chemical modification. The implications of this rather limited modification of lysine residues in the native BMA will be presented in the discussion section of this paper.

The buoyant density titration curves for native and carbamylated BMA are shown in Figure 3. The data extend from pH 5.3 to 12.7. These are for the soluble proteins only.

Table I: Amino Acid Compositions of Native and Carbamylated BMA.

	Published Values			Measured Values[d]	
	a	*b*	*c*	Native	Carbamylated
Lys	57.9	61.7	57.6	57.8	33.0
His	17.0	16.8	16.6	17.0	16.0
Arg	22.3	22.4	26.8	22.2	22.0
Asp	54.1	54.1	53.6	54.0	54.3
Thr	32.3	34.2	32.2	33.5[f]	33.5[f]
Ser	26.6	26.0	26.2	26.5[f]	26.0[f]
Glu	74.2	77.6	80.0	77.6	77.7
Pro	27.2	29.9	27.8	28.4[e]	28.6[e]
Gly	16.0	15.9	14.9	16.0	16.1
Ala	46.3	46.1	44.1	46.3	46.2
Cys	32.2	36.1	35.1	35.0[e]	35.0[e]
Val	33.4	36.5	34.5	34.4[e]	33.6[e]
Met	3.6	3.8	3.7	3.9[e]	3.7[e]
Ile	13.1	14.0	13.8	13.5[e]	13.6[e]
Leu	61.8	61.7	57.7	61.4	61.2
Tyr	18.4	20.2	19.4	20.4[f]	19.3[f]
Phe	26.4	26.7	25.5	27.0	27.1
Homocitrulline				0	25.0
Homocitrulline + Lys	57.9	61.7	57.6	57.8	58.0

[a] Stein, W. H., and Moore, S. (1949). [b] Spahr, P. F., and Edsall, J. T. (1964). [c] King, T. P., and Spencer, M. (1970). [d] Measured values are based on color yields of Gly, Ala, and Leu assuming compositions of 16, 46, and 62, respectively. [e] Values obtained after 72 hr of hydrolysis. [f] Values obtained by extrapolation to zero hours of hydrolysis.

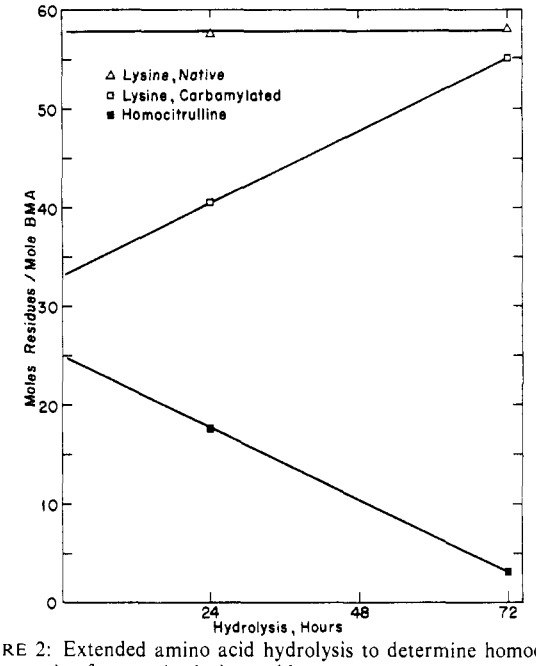

FIGURE 2: Extended amino acid hydrolysis to determine homocitrulline formation from native lysine residues.

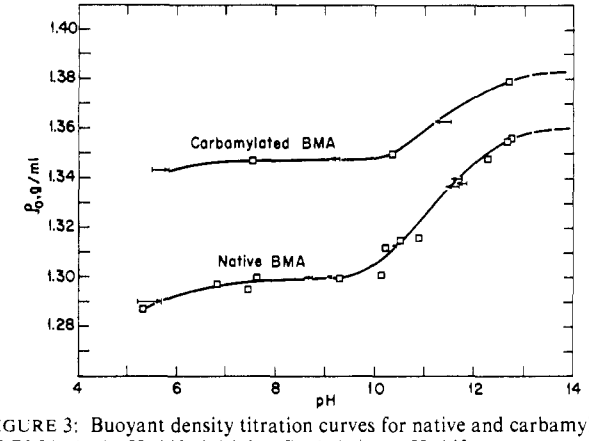

FIGURE 3: Buoyant density titration curves for native and carbamylated BMA. (→) pH shift, initial to final; (□) no pH shift.

Data for the precipitated bands near the isoelectric pH of 5.3 indicated that these values were about 0.008 g/ml above the ρ_0 for the soluble bands. Most runs were conducted at 52,640 rpm, exceptions being those made for pH 11.66, native BMA, pH 5.84, 7.50, 9.10, and 11.21 for carbamylated BMA, all of which were run at 59,780 rpm. Values of $\rho_0^0 - \rho_c^0$, the buoyant density minus the initial solution density at atmospheric pressure, were generally within ±0.015 g/ml, the greatest difference being +0.031 g/ml.

The curve for native BMA is similar in shape to the sigmoidal potentiometric titration curve obtained by Tanford et al. (1955). The curve also resembles the buoyant density titration curve of Williams and Ifft (1969), with the exception that the one obtained in this work is shifted slightly toward the right above pH 9. We cannot reconcile this difference other than to state that the two curves for the native protein were produced with separately prepared samples of BMA. The region between pH 6 and 10 displays a very gradual increase in ρ_0, followed by a sharp increase above pH 10, and then a leveling off above pH 12.5. The charge neutralization of the normally positive lysine residues causes the inflection pH of the buoyant titration curve of carbamylated BMA to increase from pH 11.2 for the native protein to pH 11.4. This compares with a value of 10.5 (Williams and Ifft, 1969) cited previously for the native protein. The curve for the carbamylated protein appears to be much flatter between pH 9.0 and 10.5 than the curve for the native material. This difference indicates an absence of normally titrating lysines, which apparently undergo ionization at a pH much lower than the pK_{int} reported below. Furthermore, the results demonstrate the preferential titration and chemical modification of easily accessible lysines in the native molecule. Hence, these residues are also the first lysines to titrate potentiometrically. Above pH 11.8 we observe that both curves increase proportionally to one another.

Analysis of the curve for the carbamylated protein reveals several contrasting features. A smaller increase in ρ_0 between pH 6 and 10 than observed for the native protein and a total increase in ρ_0 of only 0.034 g/ml as compared with 0.058 g/ml for the native BMA are readily discernible features of the titration curves. Furthermore, all values of ρ_0 for the modified protein are considerably higher than those of the native molecule at corresponding pH values. Amino acid analysis indicates that the two proteins differ only in the number of lysine residues. Therefore, the higher ρ_0 for the carbamylated mercaptalbumin over the entire pH range indicates an increase in buoyant density due solely to the modification of the lysines. The carbamylation must account for a ρ_0 difference of 0.048 g/ml at low pH and 0.024 g/ml at high pH. Assuming no conformational changes, the

latter value can only be attributed to carbamyl groups since both proteins are identically charged at pH 13. Thus, neutralization of 25 out of 58 lysine residues must account for a ρ_0 increment of 0.024 g/ml. At high pH, a residual density was observed between native and carbamylated ovalbumin (Ifft, 1971) of 0.007 g/ml. This was attributed to the carbamyl groups on 18 of the 20 lysine residues. In this work, the presence of this moiety on 25 of the 58 lysines of BMA seems to affect the buoyant density more drastically. In any case, further studies are required to ascertain the significance of the carbamyl groups in light of the different tertiary structure of these two proteins.

The work of Decker and Foster (1967) yielded intrinsic dissociation constants for the ionizable residues of BMA. These data and that of Tanford et al. (1955) indicate that the pK_{int} of the tyrosine residues in 3 *M* CsCl is about 11.5. This is in agreement with the estimated p*K* for the 10 tyrosines of ovalbumin (Ifft, 1971). The pK_{int} of lysines for most proteins have been found to fall in the pH range 9.5–10.5 (Martin, 1964), with a value of 10.7 (Decker and Foster, 1967) assigned to the 58 ϵ-NH_3^+ of native BMA. Arginine residues are assumed to titrate above pH 12.5 (Williams and Ifft, 1969), with a pK_{int} of 13.5 (Decker and Foster, 1967). Data for the buoyant titrations of poly(Lys), poly(Tyr), and poly(Arg) have been obtained (Almassy et al., 1973). From the above information and the total number of residues found in BMA, calculations of the contribution to the buoyant density of each titratable residue were made. The 58 lysines, 20 tyrosines, and 22 arginines contribute 0.004, 0.010, and 0.005 g/ml, respectively, to the ρ_0 of BMA. The total contribution for these residues titrating from pH 9.5 to pH 13.0 is about 0.019 g/ml. The calculated increment of 0.019 g/ml compares with the observed $\Delta\rho_0$ of 0.058 g/ml. Subsequent discussion indicates that the contributions of the tyrosine and arginine residues are approximately correct. The cause of the anomalous, low value for the contribution of the lysine residues is unknown at present.

Both curves in Figure 3 behave similarly above pH 11.5. The $\Delta\rho_0$ between pH 11.5 and the extrapolated value at pH 13.0 are 0.019 and 0.015 g/ml for the native and carbamylated proteins, respectively. Although the difference between the two proteins is only 0.004 g/ml, this may reflect a smaller number of tyrosines titrating above pH 11.5 in the modified mercaptalbumin. Significant changes in the titration of tyrosines were also observed in acetimidated bovine serum albumin (Avruch, 1969).

Homopolymer data show that the tyrosine and arginine residues contribute 0.015 g/ml to the $\Delta\rho_0$ observed in the pH range 11.5–13.0. If lysines account for the remainder of the total ρ_0 increment, we may assign a contribution of 0.020 g/ml for 33 lysines in the modified protein, and 0.039 g/ml for 58 lysines in the native mercaptalbumin. This corresponds to about 0.007 g/ml per lysine residue in both proteins.

Of interest here are the data obtained for the lysines of ovalbumin, which caused a ρ_0 increment of 0.018 g/ml when 18 out of 20 lysines were modified. In this work, the modification of 25 out of 58 lysines accounts for a ρ_0 increase of (0.048 − 0.024) g/ml = 0.024 g/ml. There is a definite correlation between the number of lysines carbamylated and the resultant change in the buoyant density between the two proteins. This is even more critical when one considers the different properties (*e.g.*, ion binding capacity) and tertiary structure of ovalbumin and BMA. Each modified lysine, therefore, increases the ρ_0 of the protein by approximately 0.001 g/ml.

Finally, data available for histidine residues of BMA show a pK_{int} of 6.5 for 10 imidazole groups and 7.5 for the remaining 7 groups (Decker and Foster, 1967). Ovalbumin histidines have a pK_{int} of 6.3 (Ifft and Lum, 1971). Calculations using data for poly(His) indicate a contribution to the ρ_0 of BMA of 0.004 g/ml by the 17 histidines. It can be seen in Figure 3 that there is an increase in ρ_0 of 0.006 g/ml from pH 6.0 to 8.5 for the native protein.

Hydration numbers for Cs^+ and Cl^- in going from ρ_0 = 1.300 for the native protein to 1.348 for the carbamylated molecule were calculated, using the tabulated partial specific volumes, $\bar{v}_{Cs^+}$ and $\bar{v}_{Cl^-}$ (Ifft and Williams, 1967). At ρ_0 = 1.300 g/ml, $\bar{v}_{Cs^+}$ = 19.84 ml/mol and $\bar{v}_{Cl^-}$ = 24.36 ml/mol. If 25 Cs^+ were gained by BMA as the lysines are neutralized, the resulting density would be

$$\rho_0 = \frac{67{,}000 \text{ g of protein} + (25 \times 132.9) \text{ g of } Cs^+}{(67{,}000 \text{ g}/1.300 \text{ g/ml}) \text{ ml of protein} + (25 \times 19.84) \text{ ml of } Cs^+}$$

$$\rho_0 = 1.355 \text{ g/ml}$$

The observed ρ_0 is 1.348 g/ml, and, therefore, we expect that the Cs^+ must be hydrated. Setting the above equation equal to 1.348 g/ml and adding the terms *X* g of H_2O and *X* ml of H_2O to the numerator and denominator, respectively, we calculate that 1000 g of H_2O must be added to the 25 mol of Cs^+ ions. This gives 2–3 mol of water/mol of Cs^+ gained. Alternatively, we may assume a loss of hydrated Cl^-, in which case 6500 g of H_2O must be lost with 25 mol of Cl^-. This gives about 14 mol of water/mol of Cl^-. According to the accepted hydration numbers of 3–9 for Cs^+ (Kraus, 1922) and 1–6 for Cl^- (Tanford, 1961), it would appear that the increase in ρ_0 is due primarily to a gain of hydrated Cs^+. This is the preferred explanation since few Cl^- are still bound at pH 10.

Discussion

The modification of only 25 lysine residues of mercaptalbumin under mild reaction conditions may indicate that a number of lysines are involved in binding interactions with other amino acid residues, or that the 33 unmodified lysines lie at less accessible positions in the polypeptide chain. This is in contrast to the results obtained in the acetimidation of bovine serum albumin (Avruch et al., 1969), which yielded complete lysine modification. The 43% carbamylation in the present study may be due to the substitution of a neutral residue for a charged one as opposed to the substitution of one positively charged residue by another in Avruch's work.

The limited carbamylation, then, may reflect some critical ion binding interaction in the native molecule, thermodynamically preferring a charged ϵ-NH_3^+ state to a neutral one. Vijai and Foster (1967) and Foster and Clark (1962) implicated lysine residues with the stabilization of the native structure by charge interaction with 40 carboxylate ions buried inside the protein molecule. Avruch and his associates (1969) blocked all lysines with methyl acetimidate, yielding a positively charged, but longer moiety on the lysine side chain. Their data demonstrated that carboxyl groups titrate more readily at a given pH in the modified bovine serum albumin than in the native protein. Tyrosine residues displayed the same behavior. The studies conducted by Steinhardt and Stocker (1973) corroborate these results.

The buoyant density titration curves of the native and

carbamylated BMA presented in this work offer some interesting conjectures as to the behavior of this molecule. The abnormally large buoyant density of the carbamylated protein at each pH as compared with that for the native molecule indicates that lysines must contribute 0.001 g/ml per residue to the ρ_0 increase at high pH. This may occur either directly by deprotonation or neutralization by the carbamyl groups, or indirectly by affecting the ion binding capacities of the residues that lysines may have interacted with in the unmodified state. Similar studies on ovalbumin have revealed a contribution of 0.018 g/ml to the ρ_0 due to the carbamylation of 18 lysine residues or the same value of 0.001 g/ml per residue. A conspicuous difference in the buoyant density curves of the native and modified BMA is the drastic change in the buoyant behavior between pH 9.3 and 10.5. The 25 modified lysines have increased the pH of the initial increase in ρ_0 from 9.3 to 10.4. Assuming a pK_{int} of 10.7, about 20 lysines are titrated at pH 10.4, the pH at which the ρ_0 of the modified protein begins to increase. Therefore, the modified lysines represent those in the native mercaptalbumin that are easily accessible to attack by cyanate, and they are apparently the first lysines to deprotonate. We should expect that most of the lysines have deprotonated by pH 11.7. The $\Delta\rho_0$ observed between this pH and the extrapolated pH of 13.0 indicates that there is a +0.004 g/ml difference ($\Delta\rho_0$ native $-$ $\Delta\rho_0$ carb). This may be accounted for by the delayed titration of tyrosine residues in the native protein, since it has been shown (Avruch et al., 1969) that phenolic titration occurs at a lower pK (10.3) in modified BMA than in native BMA (11.5). This abnormally high pK of native tyrosines can be accounted for by their interaction with lysine residues, which may prevent phenolic deprotonation until the ϵ-NH_3^+ groups have almost completely titrated. Based on homopolymer data (Almassy et al., 1973) the 0.004 g/ml difference would correspond to 8–10 tyrosine residues that have not yet titrated by pH 11.7 in the native protein. This correlates well with previous studies (Avruch et al., 1969) which indicated that approximately 10 residues remained protonated above pH 11 in the native protein. Finally, the possibility that a residual ρ_0 may be contributed by the carbamyl groups must be considered. Whether these groups are directly responsible for the observed $\Delta\rho_0$ of 0.024 g/ml at pH 13.0 or whether they only mediate slight conformational changes and interfere with or alter the normal ion binding behavior of BMA is not fully known at this time. Based on the data for ovalbumin (Ifft, 1971), which experienced a much smaller residual ρ_0, it seems unlikely that the carbamyl groups are responsible for an alteration in ρ_0 of such a magnitude in BMA.

Vijai and Foster (1967) have suggested that bovine serum albumin exists as two folded units of a single polypeptide chain, which undergoes an N–F transition by the separation of these units without disruption of their ordered structure. These globular portions are postulated to contain paired carboxyl and ϵ-NH_3^+ groups which are inaccessible to protons in the native state. Therefore, there is increased accessibility of carboxyl groups in the modified protein due to an expansion of the N–F type. If lysines that partake in this binding are modified, then we must assume some small conformational changes in the modified BMA. In this work we have assumed that very few of these internally associated lysines have been modified, as demonstrated by the diminished ρ_0 increment below pH 10.4. Furthermore, dichroic spectral studies of native and acetimidated bovine serum albumin (Avruch et al., 1969) have established that no major conformational changes resulted due to alteration of lysine side chains.

If indeed the interactions of lysines with carboxyl and phenolic groups characterize the native, tertiary structure of BMA, it would be of considerable interest to observe the effects of modified carboxyl groups on the buoyant titration behavior of proteins. Current research is being undertaken in this laboratory to ascertain such changes on the behavior of mercaptalbumin.

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