Perturbation of Proton and Detergent Binding Sites in Bovine Serum Albumin by Acetimidation*

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ABSTRACT: Modification of bovine serum albumin by blocking the 57 lysine residues with methyl acetimidate hydrochloride leads to a protein with identical circular dichroism spectra with that of the native macromolecule but a slightly increased intrinsic viscosity. The binding isotherm of sodium dodecyl sulfate to both protein species is identical. However, the binding-induced difference spectra are reduced in magnitude when the ligand interacts with the modified protein. Proton binding studies indicate a larger number of carboxylate and tyrosine groups are exposed in acetimidated bovine serum albumin than in native bovine serum albumin.

The lysine residues of bovine serum albumin have been implicated in stabilization of the native state through charge interaction with the 40 buried carboxylate ions (Vijai and Foster, 1967; Foster and Clark, 1962) and in the binding sites on bovine serum albumin for sodium dodecyl sulfate (Markus *et al.*, 1964). The last-named authors showed that sodium dodecyl sulfate stabilized native bovine serum albumin against urea denaturation but did not stabilize a modified form of bovine serum albumin in which the lysines were blocked to give an uncharged side chain. Tyrosine and tryptophan also have been shown to be involved in detergent binding to bovine serum albumin by Ray *et al.* (1966), Polet and Steinhardt (1968), and Reynolds *et al.* (1967) who showed spectral shifts in these aromatic residues as the result of binding C_8–C_{14} sulfate and sulfonate half-esters and C_8–C_{12} carboxylic acids to bovine serum albumin.

The object of the present work was to block all lysine residues of bovine serum albumin with methyl acetimidate hydrochloride (Habeeb, 1966; Wofsy and Singer, 1963) to form a modified protein with structure

$$P\sim NH_3^+ \longrightarrow P\sim NHC(=NH_2^+)CH_3$$

In so doing, the positive charge is left on all modified lysine residues but a longer side chain has been inserted. If ϵ-amino groups are involved in charge–charge interactions with carboxylate groups and are also part of a set of binding sites for detergent ligands, it is reasonable to expect an alteration in both detergent and proton binding properties of the modified protein. Previous studies of a limited nature (Habeeb, 1966) showed a small increase in the frictional ratio (<3%) due to acetimidation of bovine serum albumin. In this paper the hydrodynamic and optical properties of completely acetimidated bovine serum albumin are presented to show the extent of alteration of the native protein conformation due to modification. The role of lysine residues in binding of dodecyl sulfate and protons to the protein has been investigated.

Methods

Circular dichroism and optical rotatory dispersion were determined on a Cary 60 recording spectropolarimeter using 1- and 0.1-cm path-length cells and appropriate protein concentrations to maintain the dynode voltage below 4 kV. The slit was programmed to a constant 15-Å band width.

Ultraviolet difference spectra were measured in 1-cm tandem cells on a Cary 14 spectrophotometer. In all experiments reagents added to the sample cell were compensated in the reference beam.

Sedimentation velocity was determined in a Spinco Model E ultracentrifuge at a rotor speed of 52,640 rpm. The ultracentrifuge was equipped with a schlieren optical system and photographs were taken at 8 min intervals after reaching constant speed.

Viscosity measurements were made in Cannon-Manning semimicroviscometers in a thermostated oil bath held to ±0.005°. Flow times ranged from 250 to 400 sec and were reproducible to ±0.2 sec.

Equilibrium dialysis techniques for measuring the binding of sodium dodecyl sulfate to bovine serum albumin as well as the spectrophotometric method of analysis for free detergent by extraction of a methylene blue–dodecyl sulfate complex into chloroform have been described previously (Ray *et al.*, 1966; Reynolds *et al.*, 1967). All experiments were carried out at 2° in 0.033 ionic strength phosphate buffer (pH 5.6).

Hydrogen ion titration curves were obtained by continuous titration with an International Instrument Co.

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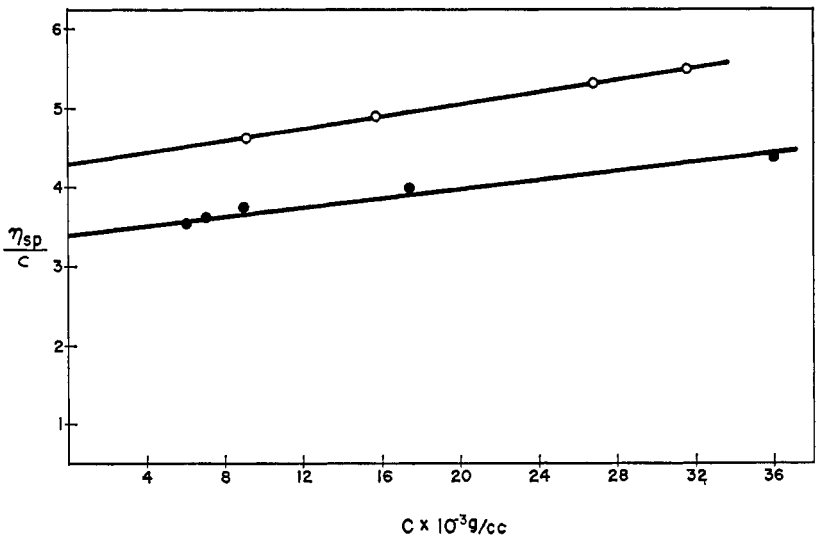

FIGURE 1: Viscosity of bovine serum (●) and fully acetimidated bovine serum albumin (○), μ = 0.033 phosphate buffer, pH 5.6; T = 25°.

difunctional recording titrator and Leeds and Northrup electrode couple 14043. A jacketed titration cell was thermostated by circulating liquid from a constant-temperature bath held to 25 ± 0.1°. All titrations were carried out at μ = 0.15 M KCl, protein concentrations ~1% by weight, and in the absence of CO_2 (flushed continuously with nitrogen). Standard KOH and HCl were used. The electrodes were calibrated with Fisher standard buffers at pH 2.0, 4.0, 7.0, and 10.0.

Fully acetimidated bovine serum albumin was prepared by three additions of 1 g of methyl acetimidate hydrochloride (Hunter and Ludwig, 1967) at 20-min intervals to a solution of 1 g of bovine serum albumin (Nutrional Biochemicals) in 100 ml of distilled water, pH 9.5, 25°. The pH was adjusted with 2.5 N NaOH after each addition of reagent and kept constant by automatic addition of 2.5 N HCl from a Radiometer automatic titrator. The reaction mixture was transferred to a cellophane bag and dialyzed exhaustively against distilled water until the pH of the retentant was constant at 5.3. The extent of acetimidation was determined by observing the rate of increase of lysine during hydrolysis of the modified protein in 6 N HCl at 110° (Reynolds, 1968). It was observed that all 57 of the ϵ-amino groups had been acetimidated. Blocking of the α-amino group was not determined.

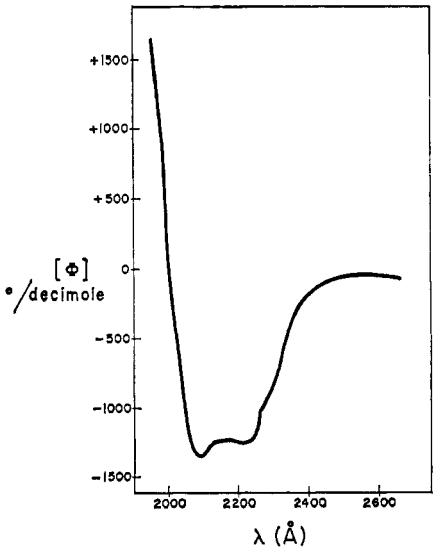

FIGURE 2: Circular dichroism of bovine serum and fully acetimidated bovine serum albumin in H_2O, 20°.

Results

Characterization of Fully Acetimidated Bovine Serum Albumin. Figure 1 presents the reduced viscosity *vs.* concentration for bovine serum albumin and fully acetimidated bovine serum albumin at 25°, 0.033 ionic strength phosphate buffer (pH 5.6). The intercept at c = 0 must be corrected for the effect of protein density (Tanford *et al.*, 1955) by addition of the term $(1 - \bar{v}_2\rho_0)/\rho_0$, where $\bar{v}_2$ = partial specific volume and ρ_0 = density of the solvent. The corrected values $[\eta]$ together with the sedimentation coefficients are given in Table I.

The small increase in $s^0_{20,w}$ on acetimidation is easily

TABLE I

	Bovine Serum Albumin	Fully Acetimidated Bovine Serum Albumin
$s^0_{20,w}$ (S)	4.42[a]	4.7 ± 0.1
$[\eta]$	3.7	4.6
M	69,000	71,430[b]
$M^{2/3}/s$	381 ± 20	376 ± 20

[a] Loeb and Scheraga (1956). [b] Calculated based on acetimidation of 57 lysine residues.

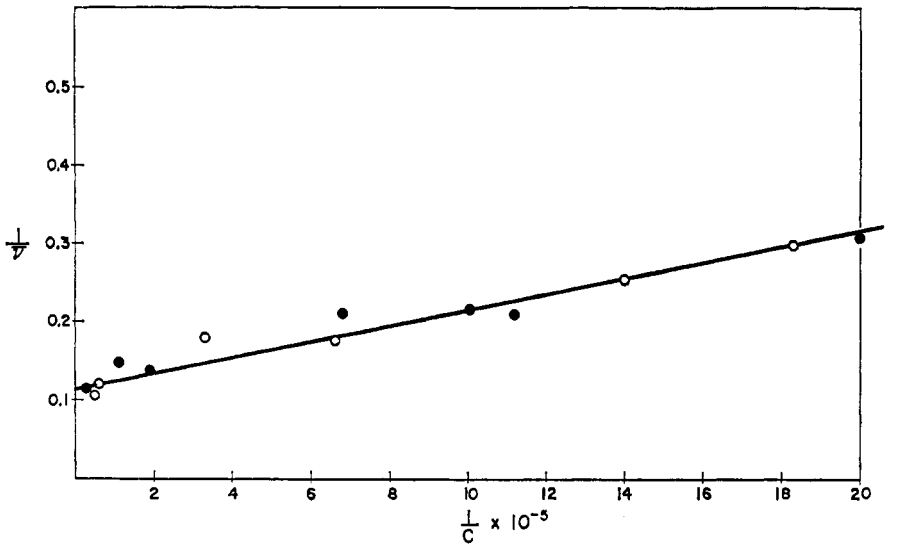

FIGURE 3: Binding isotherm of sodium dodecyl sulfate to bovine serum (●) and fully acetimidated bovine serum albumin (○) $\mu = 0.033$ phosphate buffer, pH 5.6; $T = 2°$.

accounted for by the increase in molecular weight of the modified protein.

$s = M(1 - \bar{v}_2\rho)/Nf$, where M = molecular weight, N = Avogadro's number, and f = frictional coefficient $\sim M^{1/3}$; therefore, $s \sim M^{2/3}$ assuming no change in $\bar{v}_2$. Thus the ratio of $s^0_{20,w}$ to $M^{2/3}$ is identical within experimental error for bovine serum albumin and fully acetimidated bovine serum albumin. However, the intrinsic viscosity of hydrated spheres is given by $[\eta] = \nu(\bar{v}_2 + \delta_1 v^0)$, where ν = shape factor and $\delta_1 v_1{}^0$ = degree of hydration. It is easily seen that an increase in viscosity can be counted for only by an increase in assymmetry (reflected in ν), the partial specific volume, $\bar{v}_2$, or the degree of hydration. $[\eta]$ increases approximately 6% on acetimidation of bovine serum albumin.

Figure 2 shows the circular dichroic spectra for both protein species in 0.033 ionic strength phosphate buffer (pH 5.6). There is no observable difference in this optical property suggesting no major conformational change as the result of modification of the lysine residues. $[\Phi]$ is reported as degrees per decimole of protein and is based on M_{BSA} = 69,000 and M_{AIBSA} = 71,430.

Binding of Sodium Dodecyl Sulfate to Bovine Serum Albumin and Fully Acetimidated Bovine Serum Albumin. Reciprocal plots of $1/\bar{\nu}$ *vs.* $1/C_{equil}$ where $\bar{\nu}$ is the number of sites filled and C_{equil} is the free (equilibrium) concentration of ligand are presented in Figure 3 for the binding of sodium dodecyl sulfate to bovine serum albumin and fully acetimidated bovine serum albumin. n and K which are the intercept at $1/C_{equil} = 0$ and $nd(1/C_{equil})/(d(1/\bar{\nu}))$, respectively, are identical within experimental error for both species, $n = 8$–9 and $K = 1.2 \times 10^6$.

It has been shown previously (Ray *et al.*, 1966; Polet and Steinhardt, 1968) that binding of sodium dodecyl sulfate to bovine serum albumin causes a perturbation of the tyrosine and tryptophan electronic transitions in the wavelength region 3200–2600 Å. Figure 4 shows $\Delta\epsilon$ for $\bar{\nu} = 13$ (dodecyl sulfate plus bovine serum albumin) and difference spectra at a variety of $\bar{\nu}$ values for dodecyl sulfate plus fully acetimidated bovine serum albumin. It is apparent that a much smaller $\Delta\epsilon$ results from the interaction of the detergent anion with the modified protein. As in bovine serum albumin–detergent interactions, an apparent saturation value of $\Delta\epsilon_{2860}$ corresponding with the saturation of the first set of high-energy sites (Polet and Steinhardt, 1968) is found at $\bar{\nu} = 10$.

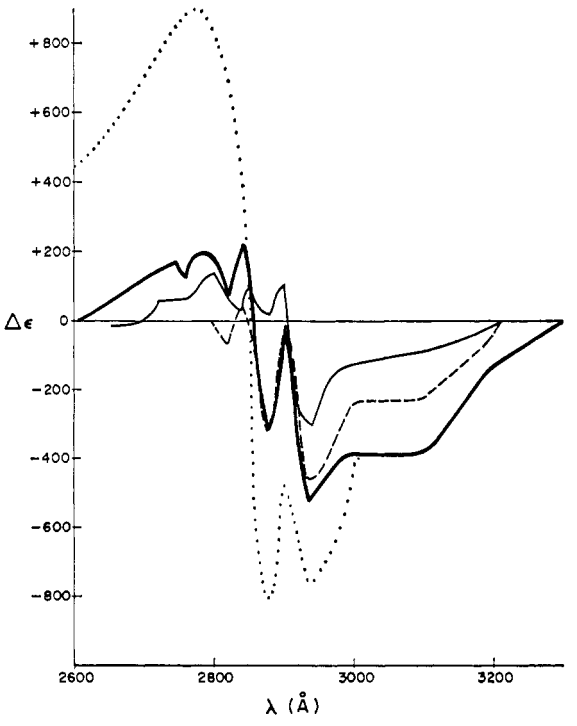

FIGURE 4: Difference spectra of dodecyl sulfate plus protein. $\mu = 0.033$ phosphate buffer, pH 5.6. (.....) $\nu = 13$, bovine serum; (———) $\bar{\nu} = 5$, fully acetimidated bovine serum albumin; (- - - -) $\bar{\nu} = 10$, fully acetimidated bovine serum albumin; (———) $\bar{\nu} = 15$, fully acetimidated bovine serum albumin.

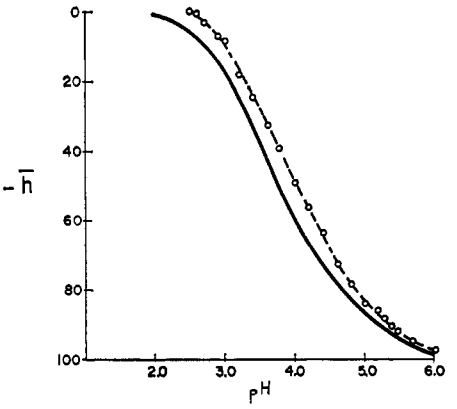

FIGURE 5: Carboxylate titration curves of bovine serum (———) and fully acetimidated bovine serum albumin (○), pH 6.0–2.0; μ = 0.15 N KCl and T = 25°.

Hydrogen Ion Titration of Bovine Serum Albumin and Fully Acetimidated Bovine Serum Albumin. Figures 5–7 show the binding of protons to bovine serum albumin and fully acetimidated bovine serum albumin at 25°, 0.15 M KCl. The data for bovine serum albumin are taken from Tanford *et al.* (1955). It is apparent from Figure 5 that the number of carboxyl residues titrated at a given pH is greater when the lysine groups are acetimidated than when they are not modified. The imidazole portion of the titration curves for both proteins is identical within experimental error of one group (Figure 6). In the modified protein (fully acetimidated bovine serum albumin) nearly all the tyrosine residues are titrated below pH 11 (Figure 7) while in native bovine serum albumin a large number of these residues remain protonated to pH 12.

Exhaustive dialysis of fully acetimidated bovine serum albumin against glass-distilled water followed by lyophilization and redissolution in 0.15 M KCl (1% protein) gave a solution of pH 5.3 as opposed to 5.6 for bovine serum albumin subjected to the same treatment. Assuming a total negative charge of 88.5 (Figure 5) plus 6 Cl^- bound (Carr, 1953) = 94.5 at pH 5.3 for fully acetimidated bovine serum albumin, the total positive charge at this pH should also be 94.5. There are 16 histidines, 57 modified lysines, and 22 arginines protonated at pH 5.3 giving a total of 95 positively charged groups.

Figure 8 gives the per cent decrease in optical rotation

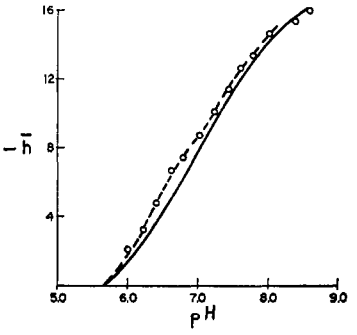

FIGURE 6: Imidazole titration of bovine serum (———) and fully acetimidated bovine serum albumin (○), pH 6.0–9.0; μ = 0.15 N KCl and T = 25°.

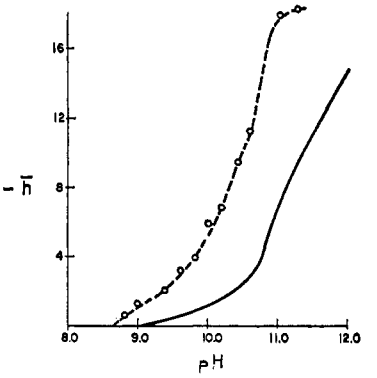

FIGURE 7: Spectrophotometric tyrosine titration of bovine serum (———) and fully acetimidated bovine serum albumin (○), pH 8.5–12.0; μ = 0.15 N KCl and T = 25°.

at 2330 Å for both proteins as a function of pH. There is a small but consistent difference between bovine serum albumin and fully acetimidated bovine serum albumin. The transition pH values of 4.5 and 7.0 are identical within experimental error for both species. However, fully acetimidated bovine serum albumin displays a larger decrease in levorotation than bovine serum albumin throughout the transitions regions.

Discussion

The identity of the circular dichroic spectra for bovine serum albumin and fully acetimidated bovine serum albumin suggests no major change in secondary structure as the result of acetimidation. However, a small increase in the intrinsic viscosity of the modified protein indicates some alteration in conformation. $[\eta]$ = 4.6 is extremely close to the value of 4.5 found for that parameter by Tanford *et al.* (1955) for the expanded form of bovine serum albumin at pH 4.0–4.5 (N–F transition) in 0.15 M KCl. Vijai and Foster (1967) have suggested that the N–F transition of bovine serum albumin is the separation of two globular-folded units of the polypeptide chain without disruption of ordered structure within the two units. The interface between these two globular portions of the protein is postulated to contain paired carboxylate and lysine residues which are inaccessible to protons in the native state. Between pH

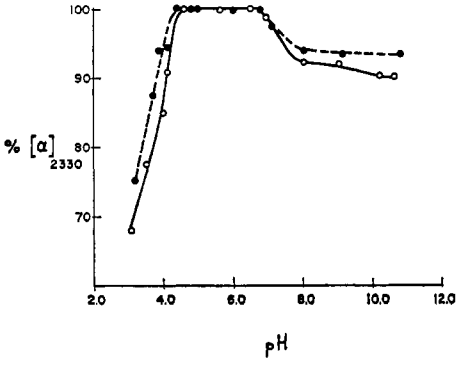

FIGURE 8: Per cent decrease in $[\alpha]_{2330}$ *vs.* pH. (○) Fully acetimidated bovine serum albumin and (●) bovine serum.

4.5 and 6.0 where no change in optical rotatory dispersion is observed for either bovine serum albumin or fully acetimidated bovine serum albumin, eight to ten more ionizable residues are protonated in the modified protein than in the native macromolecule. The entire acid titration curve is displaced toward higher pH values. These data show an increased accessibility of carboxyl groups in the modified protein which may result from an expansion of the type N–F. The increased length of the side chain attached to the lysine residues in fully acetimidated bovine serum albumin has led to an exposure of additional carboxylate groups.

The second significant difference noted between the two protein species is the reduction in the magnitude of the difference spectra due to binding sodium dodecyl sulfate to fully acetimidated bovine serum albumin. The binding isotherm itself, however, is not significantly affected by acetimidation. In addition, the tyrosine titration curve between pH 8.5 and 10.5 for fully acetimidated bovine serum albumin is significantly displaced toward lower pH values when compared with bovine serum albumin.

The binding induced difference spectra reported by Ray *et al.* (1966) and Polet and Steinhardt (1968) may be the result of a small conformation change at or near the binding site for dodecyl sulfate. In fully acetimidated bovine serum albumin a portion of this transition may have occurred as a result of acetimidation leading to a reduced effect of binding on the difference spectra.

An alternate explanation for the alterations in proton and detergent binding in fully acetimidated bovine serum albumin as compared with bovine serum albumin may be a change in local dielectric constant due to the modifying group. While it is apparent that some small conformational change has taken place in the modified protein, the mechanism by which this change affects binding properties is not easily evaluated. It is apparent that either/or both factors (conformational alterations and/or change in local dielectric constant) lead to the conclusion that the lysine residues in bovine serum albumin are involved in maintaining that tertiary structure which is characterized by masked carboxylate ions and tyrosine residues.

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