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Further Studies of the Sulfhydryl-Catalyzed Isomerization of Bovine Mercaptalbumin†

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ABSTRACT: As shown previously, bovine mercaptalbumin (N), when incubated at low ionic strength above pH 7.5, isomerizes to a new component, A, having a more positive electrophoretic mobility than N near pH 5. The reaction is sulfhydryl catalyzed. In the present study, a simple gel electrophoresis method was devised which separates A from N in a continuous pH 4.2 buffer system. Utilizing this method, the reaction was found to be faster than previously reported; the half-time at pH 8.6 and 23° in 0.01 M KCl is about 75 min. The reaction was demonstrated to be intramolecular, *i.e.*, the sulfhydryl group of the protein can catalyze reaction only within the same protein molecule. The equilibrium constant in 0.01 M KCl is near zero at pH 7.5 and rises to 0.58 at pH 8.9. KCl exerts a stabilizing effect on N and the lability of charcoal defatted bovine mercaptalbumin at low ionic strength was confirmed; in particular, prolonged (*ca.* 1 week) dialysis against water was found to result in substantial (*ca.* 10%) isomerization and denaturation of N. Evidence is presented that N and A differ covalently. Both have the same isoionic pH and acid end point. However, A binds more protons than N in the pH range of the N–F transition and fewer protons than N in the pH range of the neutral transition. A Scatchard plot of proton binding to the carboxylates of A shows no evidence for the N–F transition with all 98 carboxylates titrating with one intrinsic p*K*. The histidines of A also exhibit ideal titration behavior. Further investigations into the structural differences between N and A utilizing intrinsic fluorescence, ultraviolet difference spectroscopy, gel filtration, and susceptibility to proteolysis all show A to have a more open conformation than N at neutral pH. However, optical rotatory dispersion and circular dichroic measurements indicate N and A to have similar helix content. These results are interpreted in terms of the domain model for N.

The primary sequence of a protein is thought to contain all the information necessary to direct the folding of the polypeptide chain into a unique conformation which is stabilized by a unique set of disulfide bonds. However, Foster *et al.* (1965) considered the possibility that there might be several disulfide pairings which co-exist and give rise to the microheterogeneity observed in a population of albumin molecules. Evidence in support of this postulate was offered by Sogami and Foster (1968) and Sogami *et al.* (1969) who demonstrated that the heterogeneity of relatively homogeneous preparations of bovine plasma albumin was dramatically increased by a sulfhydryl-catalyzed "alkaline aging" reaction. The increase in heterogeneity was thought to reflect a randomization of the native set of disulfide bonds. Hagenmaier and Foster (1971) and Nikkel and Foster (1971) concluded that the aging reaction results in the formation of a new component, termed A,[1] having a positive electrophoretic mobility at pH 4.84, the isoelectric pH of native bovine mercaptalbumin. Nikkel and Foster (1971) further demonstrated the formation of A to be a function of pH and by reincubating isolated A they demonstrated the reaction to be reversible. Alkylation of the free sulfhydryl of bovine mercaptalbumin completely abolished the formation of A; however, the addition of low molecular weight thiols to the blocked sample catalyzed the formation of A. Peptide mapping studies by Nikkel and Foster suggested that the location of the free sulfhydryl did not change upon isomerization, showing the role of the free sulfhydryl group to be purely catalytic. If the reaction is a disulfide interchange it must involve at least two disulfide bonds.

This study utilized a more convenient technique of assaying for A to study the kinetics and equilibria of the reaction in more detail. A comparison of some of the physical properties of A with those of native bovine mercaptalbumin was undertaken. Since the isomerization is a function of pH and since separation of the two isomers is based on a difference in charge, particular attention was directed to a comparison of the hydrogen ion binding properties of A and the native protein.

Materials and Methods

Materials. Charcoal defatted bovine mercaptalbumin was prepared by the method of Hagenmaier and Foster (1971); all preparations had a sulfhydryl content greater than 0.9 mol/mol of protein. Other proteins utilized were crystalline bovine plasma albumin lots D71207 and G71812 from Armour Pharmaceutical Corporation, and porcine trypsin lot S-01-3

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[1] Abbreviations used are: N, the native form of bovine mercaptalbumin; A, the isomer of bovine mercaptalbumin formed by alkaline aging.

from Novo Industri A/S. Sephadex gels and ion exchange resins were from Pharmacia Fine Chemicals. Amberlite resins were obtained from Mallinckrodt, Bio-Gel P-200 from Bio-Rad, guanidine hydrochloride from Heico, Inc., and *p*-nitrophenyl *p'*-guanidobenzoate from Cyclo Chemical Co. All other chemicals were reagent grade except for those not commercially available in that grade; the highest purity available was used in those cases.

Gel Electrophoresis. The procedure of Ornstein and Davis (1964) was employed for electrophoresis at pH near 9. For separation of N and A a low pH procedure was developed. Gels were polymerized by a catalyst system reported by Jordon and Raymond (1969). The stock buffer was 0.12 M potassium citrate of pH 4.20. Stock acrylamide solution was 15 g of acrylamide and 0.6 g of bisacrylamide per 100 ml. Acrylamide was recrystallized from chloroform or decolorized with activated carbon and deionized with Amberlite MB-3. Catalyst solutions, prepared weekly, were ascorbic acid (100 mg/ml), $FeSO_4 \cdot 7H_2O$ (1 mg/ml) and hydrogen peroxide solution U.S.P. undiluted. Equal volumes of buffer and acrylamide stock solutions were mixed and polymerization was initiated by adding the catalysts in the order given above. Ascorbic acid (5 μl), 5 μl of $FeSO_4$, and 2.5–3 μl of H_2O_2 were used for each milliliter total volume of gel solution.

Before use the tops of the tubes were filled with Bio-Gel P-200 swollen in 0.035 M β-alanine titrated to pH 4.2 with citric acid. The protein solution was injected directly into the Bio-Gel slurry and mixed well. Both the cathodic (bottom) and anodic (top) buffers were 0.06 M potassium citrate (pH 4.2). A constant current of 4 mA per gel was used; initially the voltage was *ca.* 60 V but dropped to 40–45 V after about 5 min. Native bovine mercaptalbumin migrates 1–1.5 cm and A migrates 0.3–0.5 cm farther in 4 hr at 4 mA/tube.

After staining with 0.5% Naphthol Blue-Black in 7% acetic acid for at least 1 hr and destaining by transverse electrophoresis in the same solvent, gels were scanned with a Gilford 240 spectrophotometer equipped with a linear gel transport and peaks were resolved and integrated using a Du Pont 310 curve resolver.

Preparation of A. Charcoal defatted bovine mercaptalbumin was deionized by passing through Sephadex G-25 in water or by the mixed bed ion exchange method of Dintzis (1952). The deionized protein was heavily flushed with CO_2-free N_2 with stirring until the pH stabilized. After pH adjustment the flask containing the protein solution was sealed with several layers of parafilm to exclude CO_2. The solution was stored at 23° for 18–24 hr. A was isolated from equilibrium mixtures by ion exchange chromatography on SP Sephadex as described by Nikkel and Foster (1971). Carboxamidomethylated A was prepared by treating an equilibrium mixture with 6 mol of iodoacetamide/mol of protein in a pH-Stat at pH 7.0. After base uptake ceased, the protein was adjusted to 0.02 M sodium acetate–0.08 M NaCl (pH 4.70) and applied to a column of SP Sephadex. Approximately 500 ml of the low ionic strength buffer was passed over the protein bound to the resin to remove excess reagent and iodide ion and the protein was then eluted with a linear salt gradient as described in the reference.

Kinetic Studies. In the analytical method deionized bovine mercaptalbumin at 2 mg/ml was placed in a sealed vial with an access hole for a combined pH electrode. After N_2 flush with stirring for about 15 min the pH was raised to the desired value. The N_2 barrier was maintained throughout the experiment and the pH was controlled by a pH-Stat or by manual addition of base. Samples were withdrawn with a microliter syringe, and the reaction was halted by injecting the sample into the pH 4.2 stacking buffer.

A Radiometer TTT 1 Titrigraph equipped with a Radiometer GK 2026 C combined electrode was used as a pH-Stat. Unfortunately, the GK 2026 C electrode was found to leak KCl at the rate of about 10^{-6} mol/hr. To minimize changes in KCl concentration, since the aging reaction is strongly dependent on ionic strength, most aging experiments were performed in 0.01 M KCl, and the electrode was withdrawn from the solution between points after 3 or 4 hr when pH changes were small.

Measurement of Hydrogen Ion Binding. For titration studies, a Radiometer Model 25 pH meter equipped with G 222 C glass and K 100 calomel electrodes was used. The K 100 electrode was fitted with a drawn "U"-shaped capillary tip to minimize KCl diffusion. NBS standard buffer solutions of pH 4.008 and 9.180 at 25° were used to standardize the meter (Bates, 1964). The electrodes and titration vessel were encased in a well-grounded Faraday cage, which gave the instrument excellent protection from static electricity. (Drift over the course of a day was never greater than 0.005 pH unit.) Titration curves were performed by the continuous method under an N_2 barrier in a jacketed beaker thermostated at 25.0°. The experimental method and the calculation procedure followed closely those described by Nozaki and Tanford (1967). The difference in protons bound between N and A, for the titration in 0.01 M KCl, was obtained from the difference in volume of base required to reach the same pH.

Tryptic Digestion of Aged Bovine Mercaptalbumin. Digestions were carried out at 23° at a concentration of 1 mg/ml in 0.1 M KCl containing 0.001 M citrate (pH 5.00) using a 1:200 weight ratio of trypsin to substrate. Samples of 100 μl were withdrawn as a function of time, and the reaction quenched by adding 10 μl of 0.01 M *p*-nitrophenyl *p'*-guanidobenzoate in dimethylformamide and cooling to −20°. Samples were assayed by high pH gel electrophoresis on gels made with twice the normal amount of bisacrylamide, as described by Wilson and Foster (1971).

Miscellaneous Methods. The sulfhydryl content of protein samples was determined by Ellman's (1959) 5,5'-dithiobis(2-nitrobenzoic acid) reagent as used by Hagenmaier and Foster (1971). The free sulfhydryl group was alkylated by iodoacetamide as described by Wilson and Foster (1971).

Fluorescence measurements were made on a Perkin-Elmer MPF-2A fluorescence spectrophotometer. Optical rotatory dispersion (ORD) and circular dichroic (CD) measurements were made on a Cary 60 spectropolarimeter with a 6001 CD attachment.

The concentration of bovine mercaptalbumin used in titration studies was determined on a Cary 15 recording spectrophotometer. The extinction coefficient, $E^{1\%}_{278}$ of 6.67 at 278 nm (assuming a mol wt of 66,000) gave a molar extinction coefficient (ϵ_{278}) of 4.40×10^4 M^{-1} cm^{-1}. A correction for light scattering was made by subtracting the absorption at 350 nm from that at 278 nm.

Results

Resolution of N and A by Gel Electrophoresis. The high pH disc gel method of Ornstein and Davis (1964) does not resolve the N and A forms of bovine mercaptalbumin. However, a modified zone electrophoresis system at pH 4.2 gave excellent resolution of N and A. Figure 1 (inset) shows photographs of gels obtained after various aging periods. Gilford traces of

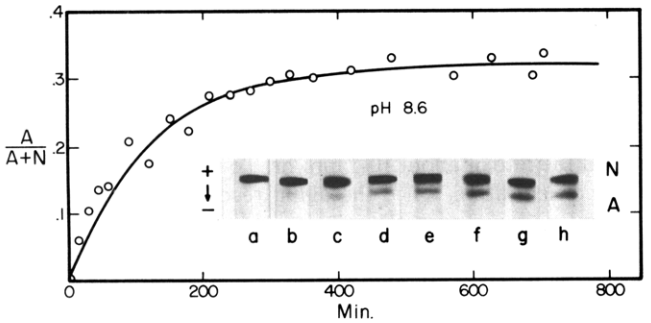

FIGURE 1: Time course of aging reaction at pH 8.6, 0.01 M KCl. The solid curve is that for a reversible first-order reaction with $K_{eq} = 0.47$ and $k_1 = 4.4 \times 10^{-5}$ sec^{-1}. Data taken from pH 4.2 gels, those shown in the inset being taken at 0 (a), 30 (b), 59 (c), 120 (d), 180 (e), 240 (f), 364 (g), and 480 (h) min.

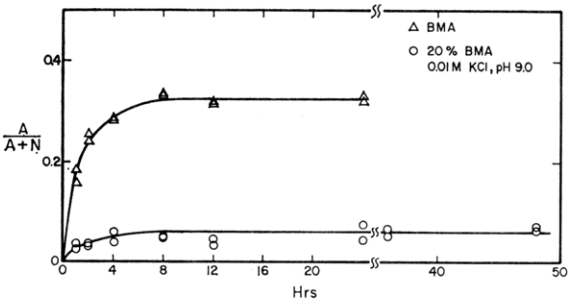

FIGURE 3: Demonstration that isomerization of N to A is an intramolecular reaction. Bovine mercaptalbumin (triangles) and a mixture of 80% blocked and 20% unblocked bovine mercaptalbumin (circles) both incubated in 0.01 M KCl at pH 9.

such patterns can be resolved unambiguously into two Gaussian peaks corresponding to N and A.

The Isomerization Reaction. The appearance (Figure 1) of a new component with a more positive electrophoretic mobility than N is in accord with the observations of Hagenmaier and Foster (1971) and Nikkel and Foster (1971) concerning the aging reaction. The solid curve in Figure 1 was calculated from the equation for a reversible first-order reaction of the type $N \rightleftharpoons A$, namely

$$k_1 t = [K_{eq}/(K_{eq} + 1)] \ln [1 - \alpha(1 + K_{eq}^{-1})] \qquad (1)$$

where $K_{eq} = k_1/k_{-1}$ = the equilibrium constant and $\alpha = [A]/([A] + [N])$ at time t. The curve corresponds to a k_1 of 4.4×10^{-5} sec^{-1} and a K_{eq} of 0.47. The excellent fit of the data to the calculated curve in Figure 1 strongly suggests the reaction to be first order and reversible.

Nikkel and Foster (1971) demonstrated that A forms N when incubated at pH 9.5. Figure 2A shows gels of a sample of isolated A incubated at pH 8.1 in 0.01 M KCl. Note that in gel a only A is present in a diffuse band. At later times an intense N band forms and increases. The squares in Figure 2B

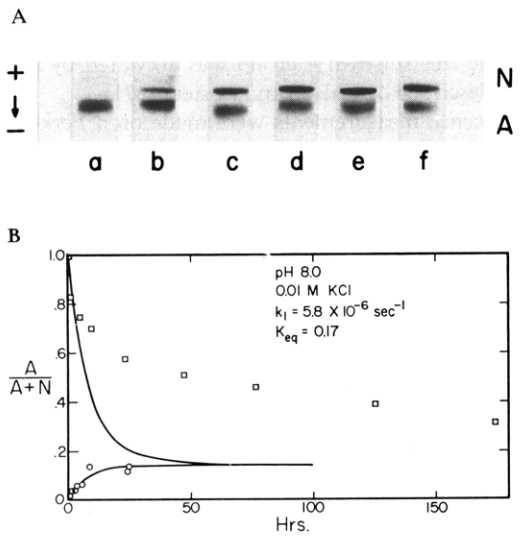

FIGURE 2: Reversal of the isomerization reaction. The pH 4.2 gels in part A show a sample of A incubated at pH 8.1 in 0.01 M KCl for 0 (a), 5 (b), 23 (c), 47 (d), 125 (e), and 174 (f) hr. The squares in part B give the data for reversal and the circles data for conversion of N to A under the same conditions. The solid curves are calculated for a reversible first-order reaction with $K_{eq} = 0.17$ and $k_1 = 5.8 \times 10^{-6}$ sec^{-1}, which best fit the data for the forward reaction.

give the quantitative results of the reversal experiment. The circles in that figure give the forward reaction at the same pH and ionic strength and correspond to $k_1 = 5.8 \times 10^{-6}$ sec^{-1} with $K_{eq} = 0.17$. Utilizing these parameters the solid curves for the forward and reverse reactions in Figure 2B were drawn. It is apparent that, although the amount of A decreases and approaches the equilibrium composition, the reversion is slower than expected.

Demonstration of the Intramolecular and Covalent Nature of the Reaction. A test was devised to determine whether or not the free sulhydryl in one albumin molecule can catalyze the reaction in another molecule. Samples of bovine mercaptalbumin and of 20% bovine mercaptalbumin plus 80% iodoacetamide blocked bovine mercaptalbumin were incubated at pH 9.05 in 0.01 M KCl at 2 mg/ml (3×10^{-5} M). Figure 3 gives the results. As expected, the bovine mercaptalbumin sample rapidly isomerized to form 35% A. However, the partially blocked sample formed only about 6% A. Even after 48 hr there is no evidence for intermolecular catalysis because fraction A does not increase beyond that expected on the basis of the mercaptalbumin content of the partially blocked sample. Therefore, the reaction must be intramolecular.

To confirm that the reaction involves a covalent alteration, a sample of A with its free sulhydryl alkylated with iodoacetamide was dissolved in 6 M guanidine hydrochloride at 23°. After 15 min the sample was renatured by removing the denaturant with G-100 gel filtration. The renatured protein was run on pH 4.2 gels with and without added N. The renatured A clearly retained the more positive electrophoretic mobility characteristic of A. Therefore, A must be a covalent isomer of bovine mercaptalbumin.

Effect of Ionic Strength and pH. The isomerization of N to A is a strong function of ionic strength and pH. To illustrate these phenomena, apparent equilibrium constants, obtained by conversion of N to the apparent equilibrium mixture, are employed. The composition was observed to be invariant for about 10 half-times once the apparent equilibrium position had been reached.

The apparent equilibrium constant K was obtained as a function of the chloride ion concentration at pH 8.9. K decreases slightly between 10^{-3} and 10^{-2} M KCl, but drops precipitously at higher chloride ion concentrations. The amount of A formed at 0.1 M KCl is immeasurably small, illustrating the tremendous stabilizing effect of KCl on the native protein.

Figure 4 shows first-order kinetic constants k_1 and k_{-1} obtained at 0.01 M KCl as a function of pH. Figure 5 gives the apparent equilibrium composition under the same conditions.

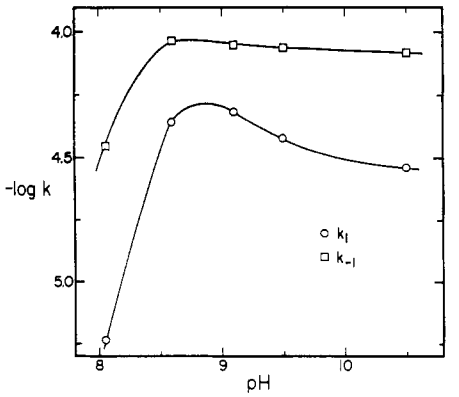

FIGURE 4: Dependence of log k_1 and log k_{-1} on pH in 0.01 M KCl.

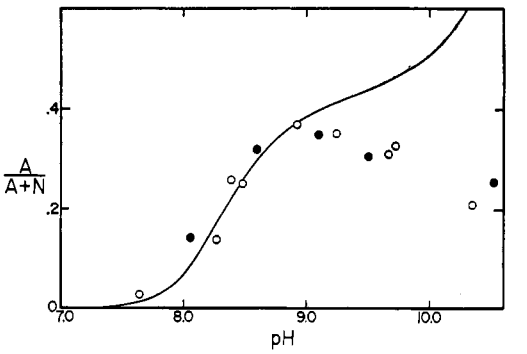

FIGURE 5: Dependence of [A]/([A] + [N]) on pH in 0.01 M KCl at apparent equilibrium. Closed circles are from kinetic experiments such as in Figure 1, open circles from apparent equilibrium constants. The solid line is a theoretical curve explained in the text.

The solid curve in Figure 5 was calculated from

$$\mathrm{d}\log K_{eq}/\mathrm{dpH} = q \qquad (2)$$

where q is the number of protons released on going from reactant to product (Lebowitz and Laskowski, 1962). For the aging reaction, $N \leftrightarrow A + qH^+$, q can be determined indirectly by separately determining hydrogen ion binding curves for N and A and taking the difference. Values for q were obtained in 0.01 M KCl and eq 2 was integrated at 0.1 pH intervals using the trapezoidal rule. The value of K_{eq} at pH 8.9, 0.58, was used as the reference to generate the curve in Figure 5 which offers a reasonable fit of the data below pH 8.9.

Hydrogen Ion Binding Properties of N and A. Figure 6 gives the hydrogen ion binding curves of N and A in 0.1 M KCl. Data points for N are omitted for clarity. Below the binding curve is given the difference in proton binding over the same pH range. Both isomers have the same isoionic pH of 5.60; however, below the isoionic pH A binds more protons than N. At still lower pH values the two curves merge, and both isomers have the same acid end point (not shown) of 93–94 protons bound to the isoionic protein. Above the isoionic pH there is another difference in proton binding with A binding fewer protons than N. The two curves merge around pH 9.0 and are identical between pH 9.0 and 10.0.

The most widely used method of interpreting hydrogen ion binding curves involves use of the equation

$$\mathrm{pH} - \log[(n - \nu)/\nu] = \mathrm{p}K_{int} - 0.434(\epsilon\psi/kT) \qquad (3)$$

where n is the number of binding sites of a particular class, ν is the number of protonated sites, pK_{int} is the intrinsic dissociation constant, ϵ is the charge of a proton, ψ is the surface potential of the protein, k is Boltzmann's constant, and T is the absolute temperature. Unfortunately, bovine plasma albumin binds anions as well as protons, complicating the estimation of the potential ψ. Hartley and Roe (1940) suggested that ψ can be approximated by the potential (ζ) which can be calculated, using the Henry equation, from the electrophoretic mobility, μ. Thus

$$\psi \cong \zeta = [6\pi\eta/Df(\kappa a)]\cdot\mu \qquad (4)$$

where η is the solvent viscosity and $f(\kappa a)$ is a function of the radius evaluated by Henry (see Abramson *et al.*, 1942). Foster and Clark (1962) have discussed the method in some detail in their study of the acid titration behavior of human mercaptalbumin, and Vijai and Foster (1967) and Decker and Foster (1967) used this approach on bovine plasma albumin.

The mobility data of Vijai and Foster (1967) and Decker (1965) span the pH range 2.6–11.5 and were used directly to evaluate the electrostatic correction factor for N. Nikkel (1970) further demonstrated that between pH 4.8 and 5.3 the mobility of A is more positive than that of N by 2×10^{-5} $cm^2\ V^{-1}\ sec^{-1}$. The data of Nikkel are in 0.03 M sodium acetate, but the same difference is assumed to hold in 0.1 M chloride. On the basis of titration data from this study, and electrophoresis experiments by Nikkel (1970), the mobilities of N and A were assumed to be the same below pH 4 and above pH 7. A mobility *vs.* pH curve for A was constructed by drawing a smooth curve from the data of Vijai and Foster below pH 4 through the data of Nikkel at pH 4.8–5.3 and merging into the data of Decker at pH 7. To convert μ to $\epsilon\psi/kT$, the factor of 7.29×10^3 V sec cm^{-2} used by Decker and Foster (1967) and Vijai and Foster (1967) was utilized for both N and A.

Foster and Clark (1962) and Vijai and Foster (1967) made use of the method of Scatchard (Scatchard *et al.*, 1957) to calculate pK_{int} and n from a single plot of the data. For the special case of equivalent carboxylate sites which interact only electrostatically, the expression is

$$(\bar{h}_{COOH}/[H^+])e^{\epsilon\psi/kT} = (1/K_{int})(n - \bar{h}_{COOH}) \qquad (5)$$

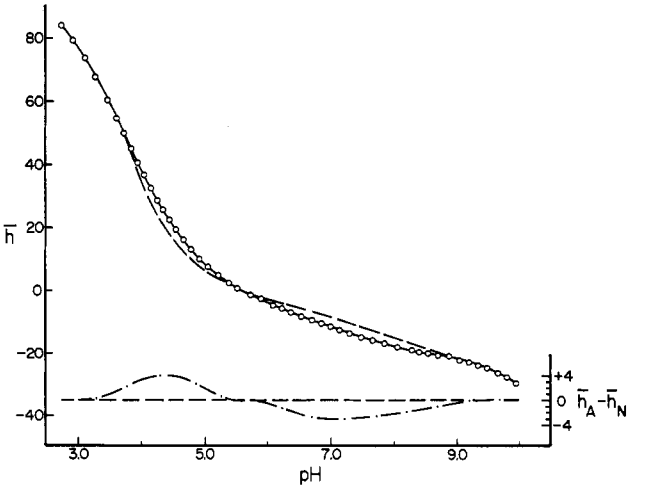

FIGURE 6: Hydrogen ion binding of N and A in 0.1 M KCl. Points for N are omitted for clarity. Lower curve (right-hand scale) is the difference in protonation over the same pH range.

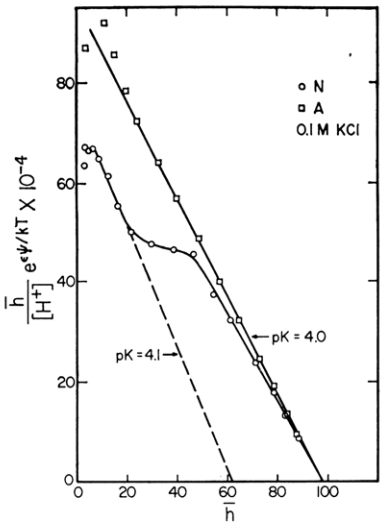

FIGURE 7: Scatchard plot, with electrostatic correction factor derived from mobility measurements, for the binding of protons to the carboxylates of N (circles) and A (squares).

where K_{int} is the intrinsic dissociation constant and $\bar{h}_{COOH}$ is the number of protons per albumin molecule bound to carboxylates. The parameter measured in titration curves is $\bar{h}$, the total number of protons bound to the isoionic protein. In the case of bovine plasma albumin, $\bar{h} \simeq \bar{h}_{COOH}$ below the isoionic pH; however, Vijai and Foster (1967) used infrared difference spectra to measure directly the number of protonated carboxylates at isoionic pH as 2.5. Decker and Foster (1967) concluded that there is 0.8 unprotonated histidine at isoionic pH. These same values of 2.5 protonated carboxyls and 0.8 unprotonated histidines were used to convert $\bar{h}$ to $\bar{h}_{COOH}$ for native bovine mercaptalbumin. The proper graphical procedure is to plot the left-hand side of eq 5 against $\bar{h}_{COOH}$. The slope of the line is $-1/K_{int}$ and at the intercept where $(\bar{h}_{COOH}/[H^+])e^{\epsilon\psi/kT}$ is zero, $\bar{h}_{COOH}$ is equal to n. To estimate the number of protonated carboxylates and unprotonated histidines at the isoionic pH for A, the hydrogen ion binding data for A were plotted using the corrected pH scale, pH + 0.434 ($\epsilon\psi/kT$) (figure not shown). Various values of the pK_{int} for carboxyls and histidines were tried. A pK_{int} of 4.0 for all 98 carboxyls and 6.5 for all 17 histidines gave a good fit of the data. These pK values yield 3.1 protonated carboxylates and 1.5 unprotonated histidines at isoionic pH, which numbers were used to convert $\bar{h}$ for A to $\bar{h}_{COOH}$. Figure 7 gives the Scatchard plots for A and N.

Further Comparative Properties of N and A. Further evidence that A exists in a more open structure than N comes from several types of experiments.

(1) SUSCEPTIBILITY TO PROTEOLYSIS. As Figure 8 illustrates, the two isomers display a marked difference in their susceptibility to proteolysis at pH 5.0. A was rapidly degraded, showing significant formation of low molecular weight fragments even within 0.5 hr; there was little or no undergraded A left after 17 hr. On the other hand, N was extremely resistant to proteolysis and even after 17 hr showed no signs of degradation.

(2) HYDRODYNAMIC VOLUME. The hydrodynamic volume of A was estimated to be 30% greater than N based on elution volumes of N, A, and the covalent dimer of N determined on the same calibrated Sephadex G-100 column and utilizing the relation of Ackers (1967).

(3) SPECTRAL PROPERTIES. The fluorescence emission spectra

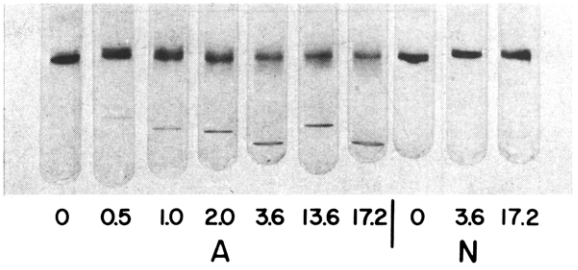

FIGURE 8: Tryptic digestion of A and N at pH 5.00 for the indicated number of hours. Samples run on high pH disc gels as described in the text.

of N and A at pH 5.00 in 0.1 M NaOAc buffer were obtained. When excited at 285 nm the native protein emits maximally at 340 nm and the aged protein at 332 nm. The relative fluorescence of N is about 50% greater than that of A at the respective maxima. The quenching of fluorescence of A can be ascribed largely to exposure of the tryptophan residues to the solvent (Teale, 1960). An ultraviolet difference absorption spectrum of A against N, obtained in 0.1 M KCl at pH 5.5, showed the spectrum of A to be blue-shifted relative to that of N, consistent with A having a more open conformation than N.

In contrast to all of the above results, ORD and CD properties indicate very little difference between N and A. Dispersion curves from 500 to 300 nm were analyzed by the Moffit–Yang equation. At pH 5.5 and 0.01 M KCl, A gave a b_0 of $-225 \pm 9°$, and N a value of $-233 \pm 3°$. Similarly the specific rotation at 233 nm differs little; for N $[\alpha]_{233}$ is $-9390°$, and for A it is $-8840°$. CD measurements in the region near 260 nm, where Velluz and Legrand (1965) have suggested disulfide bonds make a major contribution, revealed the appearance of no new bands nor the loss of any bands in the N to A reaction. However, the magnitude of the ellipticity of A was slightly less than that of N. In the far-uv, the CD spectra were also similar with both isomers having two minima at 220 and 209 nm characteristic of polypeptides in the α-helical conformation. The difference between the N and A ellipticities was less than 9% at 220 nm and about 3% at 209 nm, with N the more negative.

Discussion

The rapidity of the increase in fraction A shown in Figure 1 was unexpected; however, in the work of Sogami *et al.* (1969) and Nikkel and Foster (1971) no data were collected at times less than 24 hr. The half-time for the reaction is about 75 min at pH 8.6 and about 60 min at pH 9.0, both in 0.01 M KCl.

Several lines of evidence indicate that the aging reaction does not lead to a single, homogeneous product. Nikkel and Foster (1971) reported the irreversible formation of a component termed D which exhibited an even more positive electrophoretic mobility than A. This component was formed in high yield above pH 10. In the present study we also noted the formation of a component with more positive mobility than A. The formation of this component is apparently irreversible and is negligible up to 24 hr so that it presented no problem in the kinetic and equilibrium studies. In addition, however, the electrophoretic component identified as A is probably not homogeneous. We have observed that in general the A band is broader and more diffuse than the N band. This is clearly visible in Figure 2A. Also, electrophoretic analysis of chromatographic fractions corresponding to A in the SP-Sephadex

isolation procedure showed several minor bands of intermediate mobility. In isolating A, fractions were pooled in such a way as to minimize contamination by these components. Consequently, the isolated A does not have exactly the same composition as the A formed initially in the forward reaction. The fact that the reverse reaction rate is not consistent with the forward rate and K_{eq} is doubtless attributable to this. Also, the failure of eq 2 to fit the apparent equilibrium constant above pH 8.9 is ascribed to the existence, especially at higher pH, of multiple A forms which differ slightly in hydrogen ion binding. Since isolation of A subfractionates the aged protein, the A actually titrated would not have exactly the same titration behavior as the isomers which are in equilibrium with N. Sogami and Foster (1968) reported the extreme instability of charcoal defatted bovine plasma albumin at low ionic strength. Numerous observations confirmed their results, the most striking of which was that lengthy dialysis (*ca.* 1 week) at 2° of charcoal defatted bovine mercaptalbumin led to the formation of up to 10% D and A. Our results indicate, however, that the addition of salt has a pronounced stabilizing effect and in 0.1 M NaCl the protein appears to be quite resistant to the isomerization reactions.

The work of Nikkel and Foster (1971) clearly showed that the N → A reaction, normally catalyzed by the sulfhydryl group of the protein, can also be catalyzed by a low molecular weight thiol. It was therefore of some interest to ascertain whether the sulfhydryl group of a given protein molecule can or cannot catalyze the reaction in a neighboring molecule. The first-order character of the reaction implies that it cannot. The experiment described above on the synthetic mixture containing only 20% mercaptalbumin appears to answer this question in an unambiguous way. At equilibrium, the amount of A formed was only 20% that expected if all the molecules participated. It seems clear, therefore, that the geometry of the protein molecule is such as to preclude attack by the sulfhydryl residue of another albumin molecule.

Nikkel and Foster (1971) discussed two possible explanations for the strong pH dependence of the isomerization reaction but were unable to reach any firm conclusions because of their inability to distinguish kinetic from equilibrium effects. One obvious factor is ionization of the sulfhydryl group. If this group plays a purely catalytic role this pH dependence should enter in the kinetics only and not in the equilibrium constant.

Figure 4 shows first-order kinetic constants k_1 and k_{-1} obtained at 0.01 M KCl as a function of pH. The large increase in both constants between 8.0 and 8.6 can be attributed in the main to the ionization of the sulfhydryl group. While k_{-1} becomes essentially pH independent above pH 8.6, k_1 decreases. Obviously this cannot be due to sulfhydryl ionization, but is probably explainable in terms of the other factor considered by Nikkel and Foster (1971), namely differences in conformation between N and A.

Figure 5 gives the apparent equilibrium composition in 0.01 M KCl as a function of pH. The fraction A rises rapidly to a maximum of 37% near pH 8.9, then declines to 25% at pH 10.5. This rather peculiar pH behavior can be explained in terms of the conformational transitions which bovine mercaptalbumin undergoes above its isoionic point. Leonard *et al.* (1963) and Harmsen *et al.* (1971) have studied extensively the reversible transition which takes place between pH 7 and 9. This "neutral" transition yields a conformer, B, which we postulate to be the direct precursor of A. At higher pH values an extensive alkaline expansion takes place (Tanford and Roberts, 1952) which reduces the concentration of B, thereby reducing the forward rate constant k_1 and equilibrium constant K_{eq}.

The pH dependence of the conversion of N to A requires that N and A have different hydrogen ion binding properties. Figures 6 and 7 demonstrate that difference. In particular the Scatchard plots in Figure 7 are of interest. The Scatchard plot of N is dominated by the N–F transition which is responsible for the large discontinuity between $\bar{h}_{COOH} = 20$ and $\bar{h}_{COOH} = 45$. In agreement with the results of Vijai and Foster (1967), the N form titrates as though it has only about 60 carboxylates available for protonation with a pK_{int} of 4.1. The N–F transition exposes about 40 additional carboxylates having a pK_{int} of 4.0. On the other hand, the Scatchard plot for A in Figure 7 shows no evidence for the N–F transition. Unlike native bovine mercaptalbumin, A behaves as though all 98 of its carboxylates are available for protonation with a pK_{int} of 4.0. The histidyl titration behavior of bovine mercaptalbumin is also somewhat complex. Decker and Foster (1967) suggested two classes of histidines, 10 with a normal pK_{int} of 6.5 and 7 with a value of 7.5. Harmsen *et al.* (1971) concluded the isoionic protein to have 8 histidines with a pK_{int} of 6.9, 10 more histidines becoming unmasked and available for titration in the neutral transition. However, the titration curve for A in the neutral region is fitted to within ±1 protons assuming that all 17 histidines titrate with the same normal pK_{int} of 6.5.[2] Thus, we see that both the histidyl and carboxyl residues of A exhibit ideal titration behavior with no sign of the titration anomalies associated with the N–F and neutral transitions. In turn, the lack of transitions implies that isoionic A has a somewhat expanded conformation similar to that of N after it has undergone the N–F or neutral transition, as concluded earlier by Nikkel and Foster (1971).

The minor differences between N and A in secondary structure, as judged by ORD and CD studies, stand in striking contrast to the drastic differences in other physical properties studied. A resolution of this apparent conflict can be found in the various "domain" models for bovine plasma albumin proposed by Foster (1960), Bloomfield (1966), and King and Spencer (1970). The important feature of these models is that they envision the single polypeptide chain of bovine plasma albumin to be folded into tight globular regions connected by unstructured portions of the chain. Nikkel and Foster (1971) have demonstrated that, on aging, none of the disulfides in the amino-terminal domain form cross-links with any of the other regions, because on cyanogen bromide cleavage the "N" and "C" fragments (King and Spencer, 1970) of A can be separated without reduction of disulfide bonds. We suggest that if the conversion of N to A involves disulfide rearrangement, as seems probable, it is restricted to one of the domains. The alteration in shape of that one domain is envisioned as preventing the coalescence of the domains into the compact isoionic structure of native bovine mercaptalbumin. Consequently all amphoteric groups are exposed, thus normalizing the titration curve of A. Chromophores and fluorophores located on the interfaces of the domains are exposed to solvent and their spectral properties are altered. Much more of the peptide backbone is available for tryptic attack and the apparent hydrodynamic volume of A is increased. However,

[2] A simple calculation based on the pK_{int} values estimated for A, and assuming for N 61 carboxyls of $pK_{int} = 4.1$ together with the values suggested by Decker and Foster (1967) for the histidines lead to an estimated decrease in isoionic pH of only about 0.15 on conversion of N to A. This agrees with observations that the isoionic points of the two forms are the same within experimental precision.

the integrity of the domains and the secondary structure of the polypeptide backbone are largely unaffected.

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