A Reversible Sulfhydryl-Catalyzed Structural Alteration of Bovine Mercaptalbumin*

Harvey J. Nikkel† and Joseph F. Foster‡

ABSTRACT: Bovine mercaptalbumin undergoes an "aging" process, as previously reported for bovine plasma albumin, when stored in aqueous solution at low ionic strength and alkaline pH. In contrast to the previous conclusion that some randomization of disulfide bonding occurs, a discrete component is formed which can be separated from the native protein by moving-boundary electrophoresis and by SE-Sephadex cation-exchange chromatography. The new component has an isoelectric pH of approximately 5.2 as compared to 4.8 for the native protein and exhibits different solubility and optical rotatory properties. However, the two structures appear to be similar at very low and high values of pH. The reaction is completely inhibited when the free sulfhydryl group of the protein is blocked but can be induced in the blocked protein by an external sulfhydryl reagent. The amount of altered component formed in a given time increases sigmoidally with pH with a midpoint of pH 8.3. The reaction is reversible with an equilibrium constant of approximately unity at pH 9.5–9.7. Peptide mapping experiments indicate that the sulfhydryl group of bovine mercaptalbumin does not shift to a new position. It is inferred that the reaction involves multiple disulfide interchange, with the sulfhydryl group serving a purely catalytic role. In addition to the reversible sulfhydryl-catalyzed reaction, irreversible formation of dimer and a denatured component is observed near the above pH 10.

In order to explain the anomalous behavior of the plasma albumins with respect to the low pH N–F transition, the concept of "microheterogeneity" was proposed by Sogami and Foster (1963). This concept was also independently proposed by Štrokrová and Sponar (1963) to explain the differential stability toward thermal denaturation. In spite of the large amount of evidence supporting the microheterogeneity model, the structural basis for the molecular inhomogeneity remains obscure. Indeed, part of the observed heterogeneity is due to bound impurities (Sogami and Foster, 1967; McMenamy and Lee, 1967) and to the presence of nonmercaptalbumins in the preparations studied (Hagenmaier and Foster, 1971). However, there remain other possible contributions to the microheterogeneity (Sogami and Foster, 1968; Moore and Foster, 1968; Wong and Foster, 1969). Among the various possible causes considered earlier (Foster *et al.*, 1965), one that has received considerable attention is the possibility of variations in the disulfide pairings. Since the molecule contains 17 disulfide bonds and 1 unpaired cysteine residue, variations in disulfide pairings are conceivable. Petersen and Foster (1965) first reported a broadening of the population distribution under conditions favorable to disulfide interchange; that is, storing the protein in a solution of low ionic strength at slightly alkaline pH for an extended period of time. Sogami *et al.* (1969), in a more detailed study, reported that charcoal defatting the protein enhanced its susceptibility to this aging process. In both of these studies the evidence for increased microheterogeneity was the severe broadening of the 3 M KCl solubility–pH profiles. In both studies it was concluded that the aging reaction results from sulfhydryl-induced disulfide interchange causing a random broadening of the population distribution.

During the investigation of the possible microheterogeneity of highly purified bovine mercaptalbumin, Hagenmaier and Foster (1971) reported that a shoulder appeared in the moving-boundary electrophoresis pattern of aged fraction V bovine plasma albumin. This observation suggested that a discrete component is formed upon aging and presented an analytical tool with which the aging process could be followed. Thus, a more thorough investigation of the aging process could be initiated using the highly purified protein preparation and the improved analytical technique.

Experimental Section

Albumin Preparations. Bovine mercaptalbumin (BMA),[1] used in all experiments, was prepared according to the procedure of Hagenmaier and Foster (1971) from fraction V bovine plasma albumin (BPA) (Armour lots F-32101 and D-27309). Prior to the BMA separation using SE-Sephadex C-50 (Pharmacia Fine Chemicals, Inc.) the BPA was charcoal defatted according to a slight modification of the method of Chen (1967) as described by Sogami and Foster (1968). The SH content of BMA was routinely 0.95–0.97 mole of SH/mole as determined by the procedure of Ellman (1959) as modified by Hagenmaier and Foster (1971) employing DTNB, purchased from Aldrich Chemical Co. A molecular weight of 66,000 for BMA was used to calculate the SH titer.

The free SH group of BMA was blocked by allowing it to react with iodoacetamide (Aldrich Chemical Co.) that had

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† Present address: Chemistry Department, Illinois State University, Normal, Ill. 61761.

‡ To whom to address correspondence.

[1] Abbreviations used are: BMA, bovine mercaptalbumin; BPA bovine plasma albumin; β-ME, β-mercaptoethanol; CAM, the carboxamidomethyl group; C*AM, the [1-^{14}C]carboxamidomethyl group; DTE, 1,4-dithioerythritol; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); BPAW, a solvent consisting of 1-butanol–pyridine–acetic acid–water (30:20:6:24, v/v); BMAgd, bovine mercaptalbumin which has been aged at high pH and low salt.

been twice recrystallized from water. The reaction was carried out for 24 hr at pH 7.05 ± 0.05 at 3° using 6–7 moles of iodoacetamide/mole of BMA. The solutions were always 0.1–0.5 M in salt to prevent aging. After blocking, the solution was acidified to pH 5.2 and dialyzed extensively against water to remove excess iodoacetamide. The SH titer of the *S*-carboxamidomethyl bovine mercaptalbumin (BMA-CAM) was routinely 0.08–0.00 mole/mole. Whenever *S*-([1-^{14}C]-carboxamidomethyl) bovine mercaptalbumin (BMA-C*AM) was desired, the reaction was carried out using a sample of [1-^{14}C]iodoacetamide that had an activity of 1.08×10^8 cpm/mmole. This sample was prepared by diluting 0.5 mCi of [1-^{14}C]iodoacetamide (Amersham–Searle) with cold iodoacetamide and recrystallizing from water.

In the preparation of aged BMA (BMAgd) a solution of BMA was dialyzed repeatedly against deionized water at 3° to remove any salt. The pH of the protein solution was then raised to the desired value with 0.1 N NaOH. In order to remove any CO_2 above the surface of the solution, N_2 gas was gently blown over the solution, and the vessel was tightly sealed with parafilm. After aging for the desired length of time, the pH was lowered to 4.84 with 0.25% acetic acid or an equal volume of 0.006 M, pH 4.84 sodium acetate buffer.

Moving-Boundary Electrophoresis. A Beckman Model H electrophoresis-diffusion instrument with the standard 11-cm^3 cell (0.7609-cm^2 cross section) was used. Electrophoresis of 0.2% solutions was conducted at 3° from 4 to 8 hr. The buffer was 0.03 M sodium acetate (pH 4.82 ± 0.02) unless stated otherwise. The area under each boundary was determined by counting the Rayleigh fringes with the aid of a Nikon Shadowgraph with 10× magnification. If the components did not separate completely, the 10× magnified schlieren pattern was traced from the shadowgraph, and the peaks resolved using a DuPont Model 310 curve resolver. The averages of the mobility values determined from the ascending and descending channels were used.

Solubility–pH Profiles in 3 M KCl. The solubility–pH profiles in 3.0 M KCl were obtained by a technique similar to that described by Sogami and Foster (1968). The albumin concentration was carefully adjusted to 0.100 ± 0.003 g/ml to avoid concentration dependent variations in ΔpH_{10}^{90} and the pH midpoint.

CNBr Cleavage of BMA-CAM. A method similar to that of Gross and Witkop (1962) was used for the cleavage reaction, and the fragments were separated following essentially the procedure of King and Spencer (1970). Experimental details have been presented elsewhere (Nikkel, 1971).

Ion-Exchange Chromatography. Resolution of the N and A components, presented in BMAgd, was effected on SE-Sephadex C-50 (Pharmacia Fine Chemicals, Inc.) using a 2.5 × 44 cm jacketed column at 4° and a 0.02 M sodium acetate buffer system (pH 4.70 at room temperature) with a linear salt gradient of NaCl. A constant flow of 25 ml/hr was maintained by a peristaltic pump (Sigmamotor Inc., Model AL-2-E). The gradient components were the initial buffer consisting of 0.1 M NaCl–0.02 M sodium acetate, adjusted to pH 4.70 using glacial acetic acid, and the final buffer consisting of the same components adjusted to 0.7 M in NaCl. After the BMAgd sample had been equilibrated with the initial buffer by dialysis and applied to the column, the salt gradient was generated by using 600 ml of each buffer. Fractions of 150 drops/tube were collected and monitored at 279 mμ.

The two polypeptide chains, produced (King and Spencer, 1970) when the N fragment resulting from the CNBr cleavage of BMA-CAM was reduced and blocked with iodoacetamide, were separated on QAE-Sephadex A-25 (Pharmacia Fine Chemicals, Inc.) using a linear salt gradient at pH 8.00 (0.02 M Tris).

Optical Rotation Measurements. Optical rotation measurements were conducted at 25° with a Cary 60 spectropolarimeter employing a stoppered, water jacketed cell with a 1-cm path length. All solutions employed in the measurements were filtered through a 0.45 μ Millipore filter to minimize light scatter.

Reduction and Iodoacetamide Blocking of the N Fragment. A procedure similar to that of Bewley *et al.* (1968) was used. The reduction was allowed to proceed in 6 M guanidine hydrochloride at 37° using a 25–30:1 molar ratio of DTE (Cyclo Chemical Corp.) to cystine, assuming five disulfide bridges per N fragment (King and Spencer, 1970). After 18 hr, the temperature was lowered to 25°, and a 25% excess of iodoacetamide over DTE was added and allowed to react exactly 15 min. The reaction was quenched by adding a 1:1 molar ratio of β-ME to iodoacetamide, and the excess reagents were removed by desalting on a 2.5 × 40 cm G-25 Sephadex column with 0.001 M NH_4OH as the eluent. The reduced, carboxamidomethylated N fragment (N-R-CAM) was then lyophilized.

Tryptic Digestion. TPCK-treated trypsin (Worthington Biochemical Corp., lot no. TPTPCK 9CB) was dissolved in 0.001 N HCl containing 0.03 M $CaCl_2$. The digestion of the Asp chain was carried out at pH 8.3 and 25° under N_2 using a pH-Stat. The digestion was quenched after 24 hr by the addition of glacial acetic acid with a rotary flash evaporator. After concentration, the peptides were separated on G-25 fine Sephadex using 15% acetic acid as the eluent. The elution of the ^{14}C active peptides was monitored by liquid scintillation counting.

Peptide Maps. Peptide maps of the two ^{14}C-containing peaks in the elution pattern of the G-25 Sephadex separation of the tryptic digest of the Asp chain were prepared in the classical manner. The peptides were applied to a 46 × 57 cm sheet of Whatman No. 3MM paper as a concise spot, and electrophoresis was carried out in pH 3.5 buffer (6.6 ml of pyridine plus 66 ml of acetic acid diluted to 2.0 l.) for 60 min at 3000 V followed by descending chromatography using a one-phase solvent system of 1-butanol–pyridine–acetic acid–H_2O (30:20:6:24, v/v) (BPAW). The peptide maps were developed by staining with ninhydrin and spraying with Pauly reagent. The ^{14}C activity was monitored with a Packard Model 7201 radiochromatogram scanner.

Concentration Determination. Bovine mercaptalbumin concentrations were determined with a Hitachi Perkin-Elmer 139 UV-VIS spectrophotometer assuming $E_{1\,cm}^{1\%} = 6.67$ at 279 mμ. The concentrations of the dilute protein solutions used in the optical rotation measurements were determined with a Cary 15 recording spectrophotometer using the 0.1-OD full-scale setting. The base line was zeroed with solvent, and then the protein solution was scanned from 350 to 279 mμ. The small absorbance at 350 mμ, due to light scatter, was used as the corrected baseline to determine the actual protein absorbance at 279 mμ.

Disc Gel Electrophoresis. For standard disc gel electrophoresis, the 7% gels were made according to the CANALCO formulation of 1965, and the procedure as described by Hagenmaier and Foster (1971) was followed. For analytical purposes, the gels were scanned at 470 mμ with a Gilford

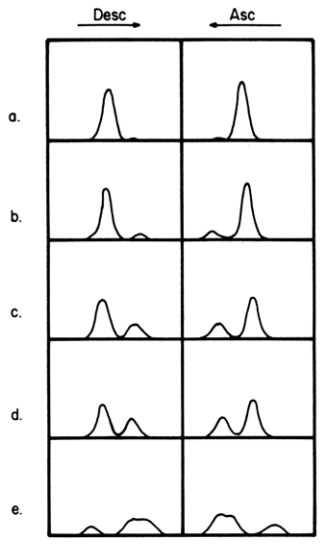

FIGURE 1: Moving-boundary electrophoresis of BMA aged for 96 hr at 3° at pH 7.06 (a), pH 8.05 (b), pH 9.00 (c), pH 10.11 (d), and pH 11.05 (e). The conditions of electrophoresis were 0.03 M sodium acetate, pH 4.82 ± 0.02, and 3°.

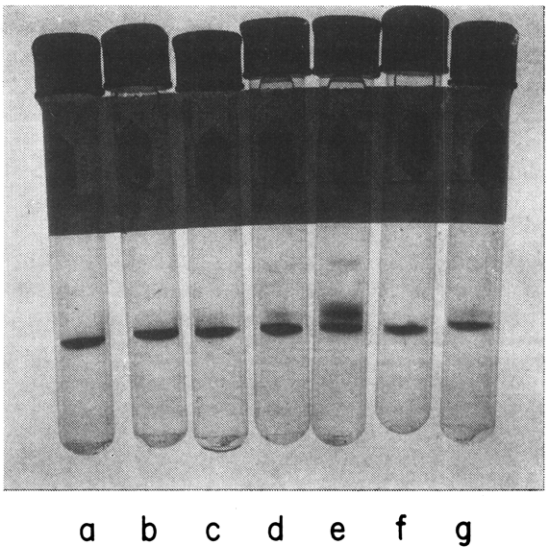

FIGURE 2: Disc gel electrophoresis of BMA aged for 96 hr at pH 7.06 (a), pH 8.05 (b), pH 9.00 (c), pH 10.11 (d), and pH 11.05 (e); N isolated from d during moving-boundary electrophoresis (f), A + D isolated from d (g).

Model 2410 automatic gel scanner, and the curves fitted with a DuPont Model 310 curve resolver.

Liquid Scintillation Counting. The sample to be counted (0.1–0.5 ml) was dissolved in approximately 20 ml of scintillation cocktail composed of 6.0 g of 2,5-diphenyloxazole (Packard Instrument Co., Inc.) dissolved in 300 ml of ethylene glycol monomethyl ether diluted to 1.0 l. with toluene. The sample was then counted with a Packard Model 3320 liquid scintillation spectrometer.

Miscellaneous. Routine pH measurements were made with a Radiometer Model 25 pH meter, equipped with an expanded scale, using a GK2026C combination electrode. The pH meter was standardized against Sargent buffers of pH 4.01, 7.00, and 10.00.

Deionized water was obtained by passing distilled water through a mized-bed ion-exchange column (Barnstead Bantam Model BD-1) and had a specific resistance of greater than 10^6 ohms cm. Dialysis bags were prepared from cellophane tubing (Union Carbide) by boiling twice in 50% saturated $NaHCO_3$, washing after each treatment, and boiling several times in deionized water at 3°. Conductivities were measured with a precision conductivity bridge made by Industrial Instruments, Inc. The conductivity cell constant was checked with a standard KCl solution. All chemicals employed, other than those specifically described above, were of reagent grade.

Results and Discussion

Dependence of Aging upon pH. Since previous studies by Sogami *et al.* (1969) indicated that the ΔpH_{10}^{90} of the 3 M KCl solubility–pH profiles of BPA increased as a function of the pH of aging, similar experiments were performed using highly purified BMA. However, moving-boundary electrophoresis and disc gel electrophoresis were used to analyze the results. The BMA solutions (*ca.* 0.34%) were aged under N_2 for 96 hr at various pH values. Moving-boundary electrophoresis experiments were than carried out at pH 4.82 where native BMA (N) has essentially zero mobility. The results in Figure 1 show a discrete, more positively charged, component which increases in amount with the pH of aging. This component will be designated as A for "aged" or "altered" albumin. In addition, there is a large amount of a third component present as a leading peak in sample e and to a lesser extent in sample d. This component will be designated as D for "denatured."

Figure 2 shows the results of the disc gel electrophoresis of the aged samples. In contrast to the moving-boundary electrophoresis results, there is only one major band in all of the gels except at the high pH values. In samples d and e, a band is seen, very weak in d and intense in e, moving slightly slower than the major band which contains N and A. This component is presumed to correspond to the third component (D) seen in moving-boundary electrophoresis. In gel e, some dimer appears as a weak band near the top of the gel. After the moving-boundary electrophoretic separation of the components present in sample d, the solution containing the N component was withdrawn from the descending channel, and that containing A + D was withdrawn from the ascending channel. The disc gel electrophoresis results of these samples are shown in Figure 2f,g. Clearly, N and A have virtually the same mobility and do not resolve during disc gel electrophoresis under these conditions, but D migrates slightly slower.

The percentages of N and (A + D) were determined by counting the Rayleigh fringes under each peak in the moving-boundary electrophoretic pattern of each sample. The percentages of D and (A + N) were obtained by fitting the disc gel scans with a curve resolver and reading the integrated values. In this way the percentages of N, A, and D present in each sample were determined. These values are plotted as a function of pH in Figure 3. The formation of A appears to be sigmoidal with a midpoint of about pH 8.3. Since SH-catalyzed disulfide interchange is generally considered to be catalyzed by the mercaptide ion (Cecil and McPhee, 1959), it is possible that the pH dependence reflects ionization of this group. However, Leonard *et al.* (1963) reported that BPA undergoes some structural modification in the pH range 7.5–9.0, and the pH dependence could reflect this conformational change.

In addition, Figure 3 shows that the amount of D com-

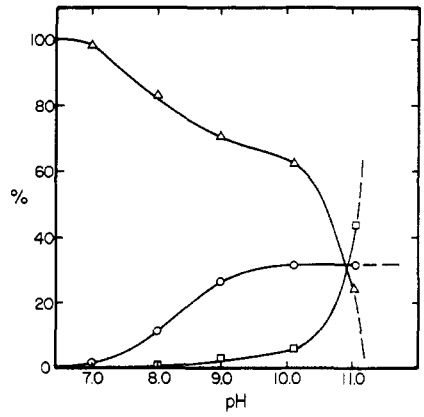

FIGURE 3: The per cent of A (○), N (△), and D (□) as a function of the pH of aging for 96 hr at 3°.

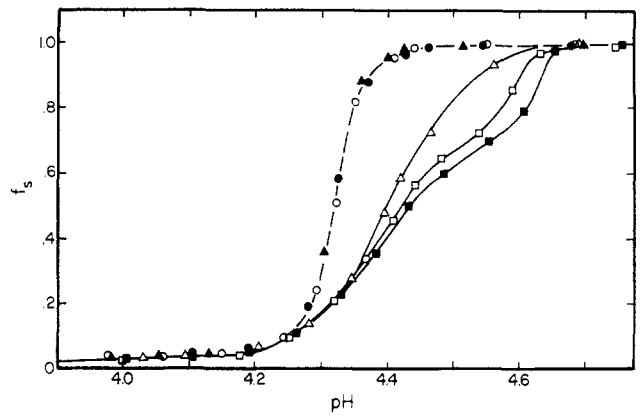

FIGURE 4: The 3 M KCl solubility–pH profiles of BMA (○), BMA-CAM (●), aged BMA (△), aged BMA-CAM (▲), BMA aged in the presence of β-ME (□), and BMA-CAM aged in the presence of β-ME (■). The aging conditions were 93 hr, pH 8.65 ± 0.05, and 3°.

ponent formed is not appreciable until above pH 10.0. It has been reported that BPA undergoes a drastic expansion above pH 10.0 (Jirgensons, 1958; Tanford and Roberts, 1952), and it is possible that the formation of the D component is related to this high pH expansion.

SH Catalysis of the Aging Reaction. It was concluded previously that the aging reaction is inhibited by blocking the free sulfhydryl group of BPA (Sogami *et al.*, 1969). Since the BMA used in this study is much more homogeneous than the BPA used in the earlier studies and since electrophoresis provides a much more sensitive criterion of aging, experiments were conducted to verify the essential role of the sulfhydryl group.

The free sulfhydryl group of BMA was blocked with iodoacetamide according to the method described earlier. The 3 M KCl solubility–pH profile of the resulting BMA-CAM, shown in Figure 4, is indistinguishable from that of BMA. This confirms the previous conclusion (Sogami *et al.*, 1969; Moore and Foster, 1968) that blocking the free SH group with iodoacetamide does not alter the conformation of BMA.

The following samples were aged at pH 8.65 ± 0.05 for 93 hr at 3°: BMA, BMA-CAM, BMA plus a 1:1 molar ratio of β-ME, and BMA-CAM plus a 1:1 molar ratio of β-ME. The 3 M KCl solubility–pH profiles of the resulting aged samples, shown in Figure 4, suggest that blocking the free sulfhydryl group of BMA completely inhibits the aging reaction. However, the sample of BMA-CAM that had been aged in the presence of a 1:1 molar ratio of an external SH reagent definitely undergoes the aging reaction, as do nonblocked BMA and BMA aged in the presence of β-ME. Disc gel electrophoresis of the previously described aged samples indicated that no dimer and only a very slight amount of D was formed. As in the samples shown in Figure 2, there was only one major band in all aged samples indicating no separation of N and A.

Moving-boundary electrophoresis experiments were performed on samples of BMA-CAM aged in the presence and absence of β-ME. As expected from the solubility data, aged BMA-CAM behaved as only one component with a mobility value that corresponded to native BMA. However, BMA-CAM, aged in the presence of β-ME, separated into two peaks corresponding to the N and A forms of aged BMA. These results show that a reactive sulfhydryl group is essential for the conversion of N to A. This group may be supplied by the protein *per se* or by an external sulfhydryl reagent.

Formation of the D Component. Since BMA-CAM does not have an available SH group to catalyze the formation of the A component, a sample of BMA-CAM was aged at pH 11.12 for 81 hr at 3° in order to determine whether the sulfhydryl group is also essential for the dimer and D component formation. The moving-boundary electrophoresis pattern of this sample at pH 4.82 indicated that, in addition to the N component, there was a peak of higher mobility corresponding to a more positively charged component (or components). However, this peak was much broader than that routinely observed for the A component. Disc gel electrophoresis of the sample indicated the presence of dimer and D component.

As stated earlier, BPA undergoes a drastic expansion above pH 10.0, and it is possible that there are more disulfide bonds exposed to the surrounding medium. Since cleavage of disulfide bonds by hydroxide ions is well documented (Andersson and Berg, 1969; Donovan and White, 1971; Kolthoff *et al.*, 1955; Stricks and Kolthoff, 1953), it is assumed that the exposed disulfide bonds are cleaved by hydroxide ions, and the liberated mercaptide ion can then initiate intermolecular disulfide interchange to form dimer or intramolecular interchange to form the D component.

Electrophoretic Separation of N and A Components. Upon examination of the disc gel electrophoresis of the aged samples (Figure 2), it is apparent that the N and A components do not separate under the conditions of this technique, whereas they are separated by moving-boundary electrophoresis. This could be due to the fact that even though N and A have different charges at low pH, they both have the same net charge and structure at elevated pH. To further examine this possibility, a sample of BMAgd (aging conditions: pH 9.3, 360 hr, 3°) was analyzed by moving-boundary electrophoresis in 0.03 M sodium borate, pH 9.32 buffer at 3°. Throughout the experiment, the protein sample migrated as only one component. This implies that N and A have similar charge and conformation at elevated pH.

A sample of BMAgd (aging conditions: pH 9.0, 96 hr, 3°) was subjected to moving-boundary electrophoresis at various pH values in 0.03 M sodium acetate buffer at 3°. The per cent of A component remained essentially constant (27.5 ± 1.5%) over the pH range of 4.86–5.32. From plots of the mobility of each component *vs.* pH, the isoelectric pH of N was determined to be 4.83 ± 0.02, and of A 5.23 ± 0.02. The average difference of mobility (μ) between A and N was 1.97×10^{-5}

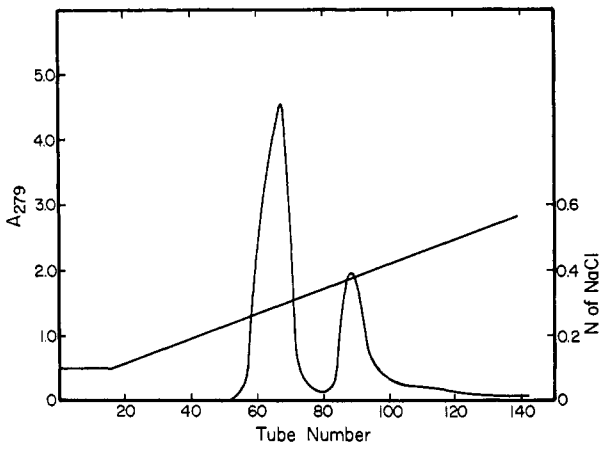

FIGURE 5: SE-Sephadex C-50 separation of A and N components of BMAgd using 0.02 M sodium acetate (pH 4.70) buffer at 4° with a linear salt gradient of 0.1–0.7 N NaCl and 150 drops/tube fractions.

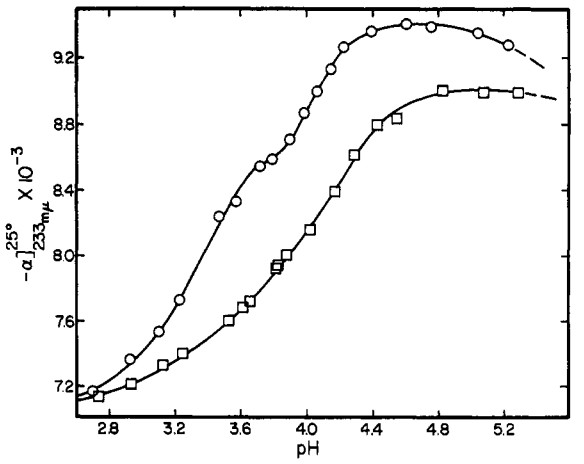

FIGURE 6: The specific rotation at 233 mμ and 25° of N (○) and A (□) as a function of pH in 0.1 N KCl.

$cm^2/(V\ sec)$. Calculations using the Henry equation and the conditions of the experiment (Hagenmaier and Foster, 1971) indicate that A carries a charge of approximately 6 units more positive than N in this pH range.

Separation of N and A Components. Since the A component is more positively charged than N at slightly acidic pH values, their separation was effected on SE-Sephadex C-50 using a constant pH (4.70), linear NaCl gradient elution system. The elution pattern of BMAgd (aging conditions: pH 9.22, 153 hr, 3°) is shown in Figure 5. There is virtually complete separation of the two species. The fractions corresponding to each peak were pooled and analyzed. Disc gel electrophoresis indicated the presence of only one band in the first peak and one major band in the second peak with small amounts of D and dimer which could be responsible for the slight trailing observed in the second peak.

The 3 M KCl solubility–pH profile of protein from the first peak was identical with that of native BMA. Moving-boundary electrophoresis indicated the presence of only one component with a mobility identical with that of native BMA. Therefore, the first peak is considered to correspond to native BMA. Thus, the second peak must clearly represent the more positively charged A component.

Optical Rotation. Optical rotation values at the 233-mμ trough of N and A components in 0.1 N KCl as a function of pH are shown in Figure 6. There is a significant difference between the two components in the pH range studied. The N form undergoes the N–F transition in the pH range of 4.3–3.7 and then undergoes the low pH expansion in the pH range of 3.7–2.7, where a final value for $[\alpha]^{25°}_{233\ m\mu}$ of −7100 is observed. This optical rotatory behavior of N is similar to that of charcoal-defatted BPA as reported by Sogami and Foster (1968).

In contrast, the A component has a less negative specific rotation than N at pH 5.3–4.7, and as the pH is lowered from 4.7 to 2.7 it shows a steady decrease in the magnitude of the specific rotation to a final value of about −7100 with no N–F transition being observed. The structural conformation present in native BMA that allows, or causes, the molecule to undergo the cooperative N–F transition appears to be lacking in the A component. It is interesting that N and A appear to have similar structures in the acid-expanded state around pH 2.7 as indicated by their identical values of specific rotation. As indicated previously, both N and A also appear to have similar charge and structure around pH 9. Thus N and A appear to differ significantly only in the intermediate pH range.

Reversibility of the Aging Reaction. Disulfide interchange reactions of low molecular weight thiols have usually been considered to be reversible equilibrium reactions. The fact that native protein can be isolated from an aged sample of BMA even after extensive aging suggests that either a portion of the molecules is highly resistant to aging, or that the reaction is a reversible process. Therefore, the samples of N and A were "reaged" and analyzed by moving-boundary electrophoresis at pH 4.82 ± 0.02. Figure 7a shows that N, isolated from SE-Sephadex, behaved as a single component. The sample of N was then allowed to reage for 96 hr at pH 9.1 and 3°. Figure 7b shows that some A component was formed during this treatment. Disc gel electrophoresis also indicated a slight amount of D and dimer formation. The moving-boundary electrophoresis pattern of the A component isolated from SE-Sephadex, shown in Figure 7c, indicates that there was some D and very little, if any, N present. (The peaks corresponding to the position of the N boundary may be due to the δ and ε boundaries.) However, when the A component was reaged at pH 9.7 for 260 hr at 3°, the N component reappeared, and the amount of D and dimer seemed to increase

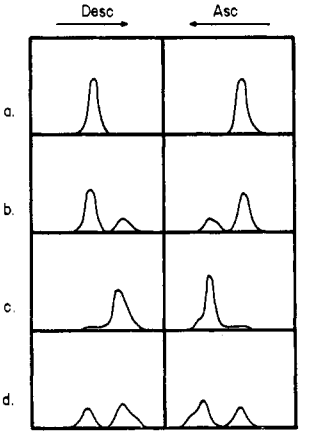

FIGURE 7: Moving-boundary electrophoresis of N isolated from SE-Sephadex (a), N reaged 96 hr at pH 9.05 and 3° (b), A isolated from SE-Sephadex (c), and A reaged 260 hr at pH 9.70 and 3° (d).

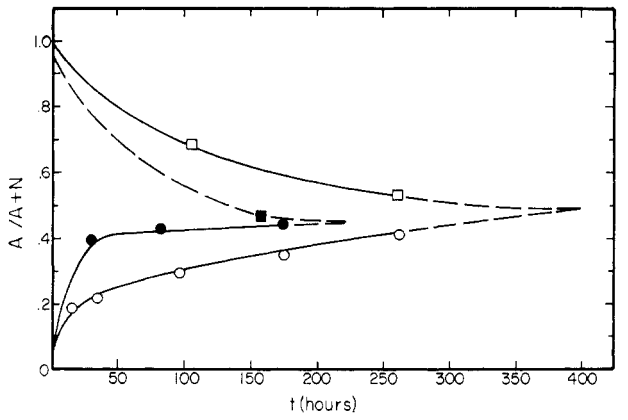

FIGURE 8: The value of $A/(A + N)$ as a function of the time of aging at pH 9.5–9.7; A aged at 3 ± 2° (□), N aged at 3 ± 2° (○), A aged at 22 ± 2° (■), and N aged at 22 ± 2° (●).

(as verified by disc gel electrophoresis) as shown in Figure 7d. This implies that the aging reaction is reversible. The solutions corresponding to the component peaks that separated in Figure 7d were withdrawn from the electrophoresis cell at the end of the run and analyzed by disc gel electrophoresis. Those results indicated that no D or dimer was present in the peak corresponding to N, and that these components only appeared in the peak (or peaks) of higher mobility.

Since the reaction is reversible, an equilibrium position should be eventually established. Samples of N and A were aged at pH 9.5–9.7 at 3 ± 2° and at 22 ± 2° for various lengths of time. The per cent of each component was determined, and the values of $A/(A + N)$ were plotted as a function of time. Since the formation of D and dimer appears to be irreversible, as indicated by the increase of these components during reaging, only the components in equilibrium were considered. However, the accuracy of the determination of the per cent of N and A was severely affected by the presence of D and dimer, and only approximate values of $A/(A + N)$ could be obtained. The results shown in Figure 8 indicate that the equilibrium constant is approximately unity at both 3 and 22°, thus implying that $\Delta G°$, $\Delta H°$, and $\Delta S°$ are all approximately equal to zero for the reaction. It is also observed that the rate of the reaction is greatly increased with an increase in temperature. The facts that the reaction is sulfhydryl catalyzed and reversible lend strong support to the idea that the aging reaction is sulfhydryl-induced disulfide interchange.

Catalytic Role of β-ME in the Aging of BMA-CAM. The simplest formulation of the reaction when the fully blocked protein BMA-CAM is aged in the presence of β-ME would result in the formation of a new SH group which should be detectable by standard analytical techniques. After aging BMA-CAM at pH 8.65 in the presence of β-ME, lowering the pH to 5.0, and removing excess β-ME by exhaustive dialysis, the SH content was only 0.012 mole of SH/mole. One possible explanation for the low SH content is that, during the dialysis to remove β-ME, the equilibrium reaction reversed, and all disulfide bonds in the protein were reformed.

To circumvent this problem, a reaction mixture of BMA-CAM plus β-ME, that had been aged at pH 9.7 for 138 hr at 3°, was allowed to react directly with a 16:1 molar ratio of [1-^{14}C]iodoacetamide at pH 8.3 in 4.5 M guanidine hydrochloride for 15 min. The 4.5 M guanidine hydrochloride was used to assure that the SH group would be exposed to the reaction medium. The excess ^{14}C reagent was removed by exhaustive dialysis, and the ^{14}C content was analyzed by liquid scintillation counting. Only 0.008 mole of [^{14}C]CH_2CONH_2 (C*AM) was incorporated per mole of BMA-CAM. Therefore, there must not be any new SH group liberated when BMA-CAM is aged in the presence of β-ME, even though the A component is formed. This suggests that the available SH group that induces the aging reaction is regenerated during the process in the tradition of a true catalyst. Consequently, the aging reaction must involve two or more disulfide bonds and liberate the original SH group. If this were the case, then one would predict that the free SH group of BMA that catalyzes the aging reaction would be regenerated in the process and remain in the same amino acid sequence along the peptide chain. However, if it is not regenerated, then the free SH group would appear at another position after aging.

Catalytic Role of the Free SH of BMA during Aging. In order to examine the possibility that the free SH of BMA is regenerated upon aging, the free SH group of BMAgd (aging conditions: pH 9.0, 142 hr, 3°) was labeled with [1-^{14}C]iodoacetamide, and the position of the ^{14}C label was compared to that of nonaged BMA. Before aging, the SH content was 0.97 mole of SH/mole, and after aging it was 0.96 mole of SH/mole, thus indicating no loss upon aging. After blocking the BMAgd with [1-^{14}C]iodoacetamide, the SH content was 0.02 mole of SH/mole, and liquid scintillation counting indicated that 0.95 mole of C*AM was incorporated per mole of BMAgd. Similar results were obtained when nonaged BMA (control) was labeled with C*AM. This implies that the ^{14}C label is specifically attached to the free SH group of the molecule. Since moving-boundary electrophoresis indicated that the aged sample contained 28% A component, if the SH group shifts upon aging about 28% of the ^{14}C label should appear at a different position in the peptide backbone.

The samples of BMA-C*AM and BMAgd-C*AM were cleaved by CNBr in 75% formic acid, and the resulting N and C fragments (King and Spencer, 1970) were separated on G-100 Sephadex. In both the aged and nonaged samples, the majority of the ^{14}C activity was found in the N fragment (residues 1–186), and the remaining activity was found in a large fragment which most likely corresponds to incompletely cleaved albumin. The N fragment of BMA actually consists of two chains, the Asp chain (residues 1–88) and the Ala chain (residues 89–186) held together by five disulfide bonds (King and Spencer, 1970). These two chains were isolated from the N-R-CAM fragments of both BMA-C*AM and BMAgd-C*AM by using QAE-Sephadex A-25. The elution patterns of the two samples were identical. The first peak eluted evidently contained the one tryptophan of the N fragment as implied from the high absorbance at 280 mμ and therefore must correspond to the Ala chain (89–186) of BMA (King and Spencer, 1970). All of the ^{14}C activity was in the second peak of both samples which means that the free SH does not shift outside the Asp chain (1–88) upon aging.

The isolated Asp chain was subjected to tryptic digestion, and the peptides were separated on G-25 fine Sephadex. The elution patterns, as monitored by liquid scintillation counting, each appeared as two peaks containing ^{14}C activity. Again, the aged and nonaged samples gave equivalent results. Analyses of the peptide maps of the first peak of both the aged and nonaged samples revealed that all of the ^{14}C activity remained bound to the point of sample application which evidently corresponded to a large peptide fragment containing the amino acid residues surrounding the free SH of BMA. The peptide maps of the second ^{14}C-containing peak contained a very small amount of ^{14}C activity at the origin, but the majority

was divided among two smaller soluble peptides. The same two ^{14}C-labeled peptides appeared in both samples, and no new labeled peptide was found in the aged sample. A possible explanation for the presence of two ^{14}C-containing peptides is that the Lys-X and Arg-X bonds surrounding the free SH group of BMA are very unsusceptible to tryptic cleavage. This would also explain the large ^{14}C-containing peptide(s) that remained bound to the origins of the peptide maps. It is possible that the free SH shifts to another cysteine residue within the same tryptic peptide. However, in view of the results when BMA-CAM was aged in the presence of β-ME, the most logical conclusion is that the free SH returns to its original position and acts as a true catalyst of the reaction.

General Remarks. In contrast to earlier conclusions (Sogami *et al.*, 1969), the aging of BMA does not result in a randomization of the disulfide bonds. Instead, there is a unique new component formed that exhibits different physical properties from the native molecule in the isoelectric pH range. However, at very low pH, where BMA exists in a highly expanded state, and at pH above 9, where the structure is also expanded or isomerized in lesser degree, N and A appear to be indistinguishable.

We have applied the diagonal technique of Brown and Hartley (1966) in an attempt to demonstrate differences in disulfide pairing between N and A. These experiments have been indeterminate because of the inherent complexity of the maps, due to the large number of disulfide groups involved plus the difficulty of attaining complete tryptic digestion. However, the facts that the reaction is very slow, sulfhydryl catalyzed, and reversible strongly indicate that it must be a disulfide interchange. A minimum of two disulfide bonds must be involved since the role of the SH group is purely catalytic. Attention should be called to somewhat related studies of Smith and Back (1965, 1968a,b) on a modified form of ovalbumin which arises on aging at alkaline pH. In that case they concluded that the difference is covalent but were unable to demonstrate a difference in disulfide pairing. It is important to note that the modification of ovalbumin was found to be irreversible.

A schematic reaction mechanism is presented in Figure 9. Between pH 7.5 and 9.0, the native BMA molecule undergoes a conformational change (Leonard *et al.*, 1963) which brings into juxtaposition the SH and disulfide bonds that participate in the reaction. These groups must also be on the surface of the molecule since they are accessible to β-ME which can catalyze the aging reaction of BMA-CAM which has no free SH. The ionized SH group can then initiate interchange between two or more disulfide bonds and be regenerated. Since the molecular transition and the proposed SH ionization both occur in the same pH range, it is not known whether the reaction midpoint of pH 8.3 reflects the expansion, ionization, or both since they could be interrelated. The A component has a permutation of disulfide bonds which does not allow it to fold into the native conformation when the pH is lowered. Consequently, the A form exhibits the different charge, solubility, and optical rotation properties that are observed. The high pH expansion which occurs above pH 10.0 probably exposes more disulfide bonds which are susceptible to attack by hydroxide ion, resulting in the formation of D and dimer. This latter reaction may be related to the OH^--catalyzed cleavage of a BPA dimer in which case Andersson (1970) has shown disulfide interchange to be involved.

These results are of particular interest with respect to the "thermodynamic" concept that the three-dimensional structure of a protein is solely dictated by the primary structure and

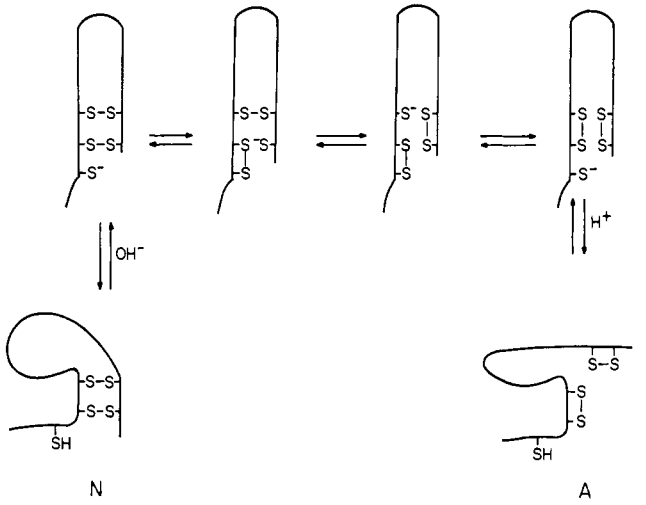

FIGURE 9: The proposed reaction mechanism for the aging of BMA.

that there should be a single configuration of disulfide bonds dictated by the most stable conformation. Anfinsen (1962) has presented evidence that one arrangement of disulfide bonds is favored in ribonuclease. However, the situation is more complex in BMA which is much larger and contains more disulfide bonds. In light of the evidence presented in this paper, it appears that the primary structure of BMA dictates at least two different three-dimensional structures with different disulfide pairings and that these two structures are equally stable at pH near 9.5 under the condition of these experiments. Studies currently in progress in our laboratories are directed at the determination of the actual chemical differences between N and A and of their relative thermodynamic stabilities under more nearly normal physiological conditions.

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γ–γ Cross-Linking Sites in Human and Bovine Fibrin*

Renné Chen† and Russell F. Doolittle‡

ABSTRACT: The amino acid sequence of the tryptic peptides involved in the γ–γ cross-linking of human fibrin has been studied. Comparison with the previously reported bovine sequences indicated two amino acid differences in the acceptor peptide. One of these changes, a histidine/glutamine change, occurred at position 10, a residue we previously thought was the acceptor half of the ϵ-(γ-glutamyl)lysine cross bridges. Accordingly, a reexamination of the bridge position in the bovine γ–γ cross-linking unit was also undertaken. The sequence of the bovine acceptor peptide was confirmed, but experiments with a radioactive substitute donor (glycine ethyl ester) indicate that the reciprocal cross bridges in both species involve the glutamine residues at position 7. As a consequence, the bridges which link the overlapping antiparallel chains are actually eight residues apart instead of the five previously reported. Characterization of the adjacent carboxy-terminal peptide has revealed it to be a pentapeptide.

The transformation of vertebrate fibrinogen molecules into a fibrin gel is a spontaneous self-assembly process following upon the removal of the fibrinopeptides by thrombin. The resulting gel, which is held together by a variety of weak forces and cooperative effects, is readily dissolved by dispersing agents such as concentrated urea or guanidine solutions. Under appropriate conditions, and certainly *in vivo*, the gelation can be reinforced by a transamidase-catalyzed introduction of ϵ-(γ-glutamyl)lysine covalent bonds. Fibrin gels containing significant numbers of these cross-links are no longer dispersable in urea or guanidine solutions. A series of reviews dealing with various aspects of fibrinogen and fibrin chemistry has recently appeared (Blombäck and Blombäck, 1970; Doolittle, 1970; Loewy, 1970; Lorand, 1970; Mihalyi, 1970; Pisano *et al.*, 1970).

Recently we reported the occurrence of two distinct bridging systems in cross-linked fibrin involving different constituent polypeptide chains, although in both cases presumably employing ϵ-(γ-glutamyl)lysine bridges (Chen and Doolittle, 1969). In one system, γ chains are dimerically[1] linked to other γ chains (Chen and Doolittle, 1969, 1970; Takagi and Iwanaga, 1970). In the second kind, we initially thought that γ chains were hooked to α chains. McKee *et al.* (1970) subsequently have shown that the latter system actually involves only α chains; in contrast to the γ–γ dimers, the α-chain cross-linking process continues at a slow but persistent rate to the formation of multimers.

The γ–γ cross-linking unit consists of overlapping antiparallel carboxy-terminal γ-chain segments which are reciprocally bridged. Originally, we reported that the ϵ-(γ-glutamyl)lysine cross-links were situated only five residues apart in cross-linked bovine fibrin. At the time, however, we noted that there were at least two amino acid replacements in the corresponding human peptides. Completion of the human amino acid sequence in this region has revealed that one of these changes is the replacement of the glutamine residue (glutamine-10) we had supposed was the acceptor portion of the cross-links. As a result we have reinvestigated the bovine structure also and now report that, although the proposed amino acid sequence of the bovine acceptor peptide was indeed correct, the principle site of incorporation of a radioactive substitute donor is at glutamine-7 in both species, indi-

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† Present address: Experimental Therapeutics Branch, National Heart and Lung Institute, Bethesda, Md. 20014.

‡ Career Development awardee, U. S. Public Health Service; to whom to address correspondence.

[1] It has been pointed out to us that the existence of γ–γ dimers was first suggested by Wilkinson (1967).