Spin-Label Studies of the Sulfhydryl Environment in Bovine Plasma Albumin. 2. The Neutral Transition and the A Isomer†

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ABSTRACT: Since we were able to demonstrate that the sulfhydryl group is located in the crevice which opens during the N-F transition (Cornell, C. N., & Kaplan, L. J. (1978) *Biochemistry 17* (preceding paper in this issue)), the investigation was extended by characterizing the environment during the N-B transition and in the A isomer. The results indicate that the N-B and N-F transitions are very similar in that the sulfhydryl group moves from a restricted to unhindered environment during both. The use of the molecular dipstick technique further demonstrated the similarity between the F and A forms. However, since A is a covalently stabilized form of albumin after a pH dependent transition, it retains its properties during subsequent pH changes rather than reverting to the N form. We were thus able to titrate spin-labeled A through the pH range of the acidic transitions without detecting the N-F transition. Isoelectric focusing analysis of A generated during alkaline aging and purified by SP-Sephadex chromatography indicates that it is a mixture of a small number of albumin forms rather than the large number of components once thought to be formed during aging.

In the preceding paper we presented the results of our spin-labeling studies of the environment of the sulfhydryl residue during the conformational transitions of bovine plasma albumin which occur on the acidic side of the isoionic pH (Cornell & Kaplan, 1978). The details of the spin-labeling technique and the advantage of its application to a study of this type were reviewed. Besides monitoring the acidic transitions, the sulfhydryl environment in the native state of both bovine and human albumin has been determined by the molecular dipstick procedure of spin labeling (Hull et al., 1975; Cornell, C. N., Chang, R., and Kaplan, L. J., submitted.)

In addition to the acidic transitions, both bovine and human plasma albumins have been shown to undergo a pH dependent conformational change in slightly alkaline solution (Leonard et al., 1963; Jirgensons, 1959). This transition, known as the neutral (or N-B) transition, involves a cooperative change in the tertiary structure of the molecule without any significant change in helical content. Since Harmsen and co-workers (Harmsen et al., 1971) showed that calcium ion shifts the transition to lower pH and affects the affinity of BPA[1] toward hydrogen ions in a manner analogous to the Bohr effect seen with hemoglobin, this transition has taken on substantial interest with respect to the physiological role of the albumins.

Plasma albumin also undergoes an aging process when stored at low ionic strength at alkaline pH. The aging process is catalyzed by the free sulfhydryl group and involves sulfhydryl-disulfide interchange which results in the conservation of the sulfhydryl at its origin position. This process was thought to result in a randomization of disulfide bonds in a nonspecific manner (Sogami & Foster, 1968) until recent studies (Nikkel & Foster, 1971; Stroupe & Foster, 1973) have shown that the aging process is more specific. The protein component A formed during this process has a more open conformation than N at neutral pH and does not undergo the N-F transition. More recent studies by Wallevik (1976) indicate the physiological importance of this process as the first step in the in vivo catabolism of albumin.

This study was undertaken to monitor the environment of the sulfhydryl residue during the N-B (neutral) transition and to determine the nature of its environment in the A isomer. Since the transition is similar to the N-F transition and since A is thought to have a structure similar to F, particular comparisons will be made between these transitions.

Experimental Section

Preparation of Albumin Solutions. The bovine plasma albumin (fraction V, Sigma lot 74-C-0253) was defatted by the method of Chen (1967) as modified by Sogami & Foster (1968). Care was taken so that the pH of albumin solutions stored in the cold was not higher than 5.5. The salt present from the charcoal treatment was not removed until just before use to further decrease the possibility of aging.

Preparation of "A". A was prepared from charcoal-treated BPA according to the method of Stroupe & Foster (1973). The 10% albumin solution was desalted by extensive dialysis against distilled, dionized water and then was incubated at pH 8.90 for 24 h at room temperature. The aging reaction was stopped by lowering the pH to 4.75. The solution was then dialyzed against the buffer to be used during the separation of N and A, i.e., 0.02 M sodium acetate, 0.1 M sodium chloride, pH 4.75.

Separation of N and A Isomers. Separation of the N and A isomers was achieved according to the method of Nikkel & Foster (1971) on SP-Sephadex G-50 (Pharmacia Fine Chemicals, Inc.) using a 2.5 × 44 cm column equilibrated at 4 °C. After pouring and equilibrating the Sephadex column with pouring buffer (0.02 M sodium acetate, pH 4.75, 0.1 M sodium chloride), the dialyzed aged PBA sample was applied. The components were then eluted with a linear salt gradient formed by an initial buffer (the pouring buffer) and a final buffer consisting of the pouring buffer adjusted to 0.7 M NaCl. Fractions of 20 drops/tube were collected and the eluent was

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[1] Abbreviations used: BPA, bovine plasma albumin; N, the normal conformer of bovine plasma albumin existing in the isoionic pH range; F, the conformer existing in the pH 3.5-4.0 range; B, the basic conformer arising in the neutral transition; A, the isomer formed by alkaline aging; ESR, electron spin resonance; MSL I, II, III, IV, and V, the maleimide spin labels whose structures are shown in Figure 1 of Cornell & Kaplan (1978).

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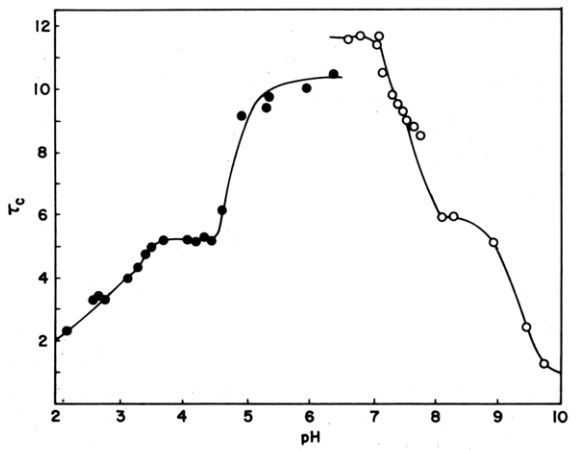

FIGURE 1: Rotational correlation time in nanoseconds for albumin, labeled on the sulfhydryl with MSL III, titrated through the acidic (filled circles) and basic (open circles) transitions.

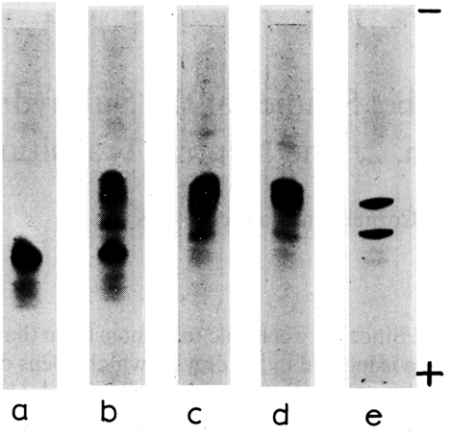

FIGURE 2: Isoelectric focusing gels of pH 4–6 range. (a) BPA; (b) the mixture of albumin forms after alkaline aging; (c) the A form after isolation on SP-Sephadex; (d) the A form prepared by H. J. Nikkel; (e) β-lactoglobulins A and B (for comparison).

monitored at 279 nm. The fractions obtained from the chromatography were analyzed by isoelectric focusing on polyacrylamide gels (Kaplan & Foster, 1971) instead of the low pH electrophoresis procedure described by Stroupe & Foster (1973). The samples containing A were pooled and concentrated to approximately 10% by ultrafiltration using Amicon PM-10 membranes.

Spin Labeling the Albumin and A Isomer. The albumin solutions were reacted with the various nitroxide spin labels containing a maleimide attaching group as previously described (Cornell & Kaplan, 1978). The A aliquots, used in the molecular dipstick experiment, to which the various spin labels (MSL I through V) had been attached were dialyzed against a 0.1 M sodium phosphate buffer, pH 6.4. The A spin labeled with MSL III to be used in the acidic titration experiment was dialyzed against 0.1 M NaCl. The albumin spin labeled with MSL III to be titrated though the neutral transition was dialyzed against 0.154 M NaCl, 0.002 M $CaCl_2$ which are the conditions reported by Zurawski & Foster (1974).

Miscellaneous Methods. Various procedures including those for titration of spin-labeled protein, the determination of protein concentration, sulfhydryl titer, and ESR spectra were as previously described (Cornell & Kaplan, 1978). The isoelectric focusing on unwashed polyacrylamide gels was as described by Kaplan & Foster (1971) employing pH 4–6 carrier ampholytes from Bio-Rad (Bio-Lyte, lot 13973).

Results and Discussion

N–B Transition of Sulfhydryl Spin-Labeled Albumin. Since the intermediate length spin-label MSL III which presumably projects to the lip of the sulfhydryl crevice yielded clear results for the acid transitions, this spin label was employed to study the neutral transition as well. On the right side of Figure 1 is shown the rotational correlation time for spin-labeled BPA titrated through the N–B transition and to higher pH. The τ_c was calculated using the dominant high- and low-field peaks as well as the center line at each pH according to the procedure of Smith (1972). A substantial change in the environment of the sulfhydryl is evident from the large change in τ_c.

For the part of the curve representative of the N–B transition, a cooperative expansion with a midpoint of approximately pH 7.8 is obtained in good agreement with the data of Zurawski & Foster (1974). Above pH 9 the molecule appears to be undergoing further unfolding as indicated by the decreasing τ_c. From these data (Figure 1) the similarity between the acid and alkaline transitions is striking. In both cases the sulfhydryl appears to undergo two consecutive changes in environment leading to a high degree of exposure.

A number of other studies have attempted to determine the state of the sulfhydryl group under the above conditions. Williams & Foster (1960) studied the perturbation of the ultraviolet spectrum of anthracene coupled to BPA through the sulfhydryl. From pH-dependent changes in the differential spectrum, they concluded that the anthracene chromophore is more enfolded in the globular structure of the protein around pH 6 than in the expanded structures at lower or high pH. In a study of the changes in optical rotatory dispersion of BPA during the alkaline transition, Leonard et al. (1963) also concluded that there is an unmasking of hydrophobic regions which result in an increased freedom of rotation of side chains immobilized in the native structure. They also noted the similarity of the neutral transition to the N–F transition. The opposite conclusion was reached by Goldsack & Waern (1971) from pressure jump studies. They interpreted the optical rotation data as being due to a folding of an exposed hydrophobic pocket and, in conjunction with their data hypothesized that the sulfhydryl group was involved in this folding process, although they offer no direct evidence for the involvement of this group.

Finally, Zurawski & Foster (1974) monitored the environment of the sulfhydryl group through the neutral transition employing ^{19}F nuclear magnetic resonance spectroscopy after introducing a sulfhydryl specific fluorinated probe. Unfortunately, they were unable to deduce the exact nature of the environmental changes, stating that they could not be represented by a simple change in exposure to solvent. They concluded only that the transition results in "some alteration in the local environment of this group" and speculated as to the causes of this alteration. In a more general vein, they noted the similarity of the shift in magnetic resonance signal on going from the N form to either the F or B states.

Preparation and Characterization of A. The A obtained from the SP-Sephadex column was heterogeneous as is clearly shown in the isoelectric focusing gels (Figure 2). The sulfhydryl content of this pooled sample was approximately 60% indicating some nonmercaptalbumin components. These results confirm the suspicion of Nikkel & Foster (1971) and Stroupe & Foster (1973) that A is not a homogeneous product of the aging process. However, since this is the sample characterized by these workers, no further purification was performed and this A preparation was spin labeled.

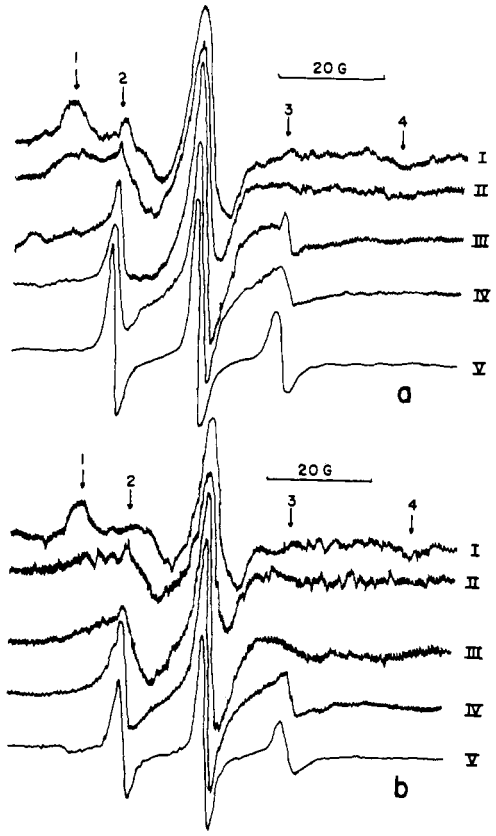

FIGURE 3: ESR spectra of different forms of albumin. Arrows 1 and 4 indicate the location of the low- and high-field peaks that are characteristic of a highly immobilized nitroxide radical. Arrows 2 and 3 indicate the analogous peaks for a freely rotating radical. I–V designate the various spin labels. (a) A; (b) F.

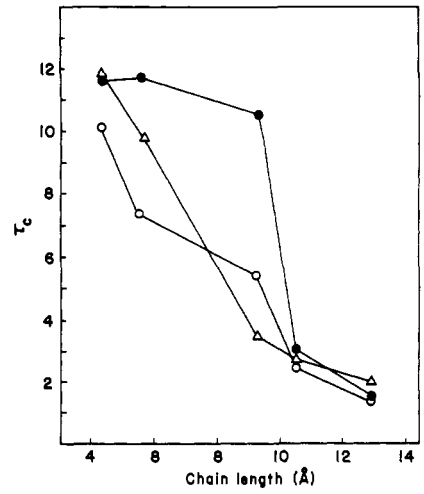

FIGURE 4: Rotational correlation time in nanoseconds vs. chain length of the spin labels. (Filled circles) N; (triangles) A; (open circles) F.

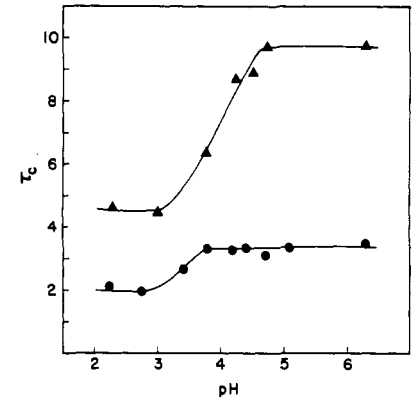

FIGURE 5: Rotational correlation time in nanoseconds for A titrated through the acidic transitions. (Filled triangles) Labeled with II; (filled circles) labeled with III.

Spin-Labeled Sulfhydryl Residue in A. The spectra of the series of maleimide spin labels attached to A are shown in Figure 3a. With the short spin labels (MSL I and II) a peak representative of a freely rotating nitroxide is evident even though there is large contribution from a restricted nitroxide. For the longer spin labels (III, IV, and V) bound to A, the spectra are those of nitroxides in fairly nonrestrictive environments. From the spectra for N (see Figure 1a of Cornell & Kaplan, 1978) and F (Figure 3b), it can be seen that the sulfhydryl is relatively more restricted in N than either F or A and slightly more restricted in F than A. This can be seen in a more quantitative way by comparing the τ_c values for each form. Figure 4 shows that while N exhibits a sharp break, A does not, exhibiting behavior very similar to F. These molecular dipstick results indicate a restrictive environment for the sulfhydryl in N and a freer (perhaps funnel-shaped crevice) environment for A and F.

Acid Titration of Spin-Labeled A. In an attempt to further substantiate the similarity of the sulfhydryl environment in A and F, A was titrated through the region that normally exhibits the N–F transition. Figure 5 shows this titration for A spin labeled with MSL II and III. MSL III attached to A was too freely rotating to exhibit very much of a change through this pH range. However, with MSL II a gradual sigmoidal change in τ_c was observed as the pH was lowered but there was no evidence for an N–F conformational change. (For comparison native albumin labeled with MSL II shows a characteristic N–F transition as shown in the preceding paper in Figure 4.) This indicates that the conformational structure of native BPA responsible for the N–F transition is missing and the sulfhydryl is in a less hindered environment.

Further Discussion. The results of this investigation are in general agreement with the work of Foster and coworkers regarding the N–B transition and the properties of the A isomer. The present study also shows that during this transition the sulfhydryl group moves from a restricted environment to an environment of relative freedom where it can catalyze the disulfide interchange postulated by Nikkel & Foster (1971) and form the isomer demonstrated by Wallevik (1976) to be physiologically important.

During this investigation on the location of the sulfhydryl we tried to reconcile our results placing the sulfhydryl in a restrictive environment with the amino acid sequence results (Brown, 1975) and the domain models (see review by Peters, 1975) which place the sulfhydryl near the amino terminus of the polypeptide chain of albumin in a region itself not restricted by disulfide bonds. Of course the linear sequence gives no indication of the location of a residue in the globular structure and one can only speculate that at neutral pH the sulfhydryl is in a restricted (yet from all indications, highly reactive) environment near a cluster of hydrophobic and neutral residues (Ohkubo, 1969). The crevice is also presumably lined with ion pairs such that with a decrease or increase in pH the molecule undergoes specific conformational transitions followed by additional unfolding and expansion at more extreme pH. One net effect of all of the conformational transitions is the emergence of the sulfhydryl from a restrictive environment to a relatively free one.

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Spin-Label and Deuterium Order Parameter Discrepancies in Bilayers: One Possible Explanation†

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ABSTRACT: We have simulated electron spin resonance spectra of anisotropically immobilized spin labels of the type seen in lipid and soap-like bilayers using a rigorous formalism which explicitly includes the effects of spin-label motion. In most bilayer systems, spin-label experiments have shown lower order parameters then deuterium-label experiments. In the past this apparent decrease in the order parameters was thought to reflect the distortion of the bilayer by the doxyl ring of the spin probes. We wish to report that this type of discrepancy may be due to the neglect of important motional effects in the time-independent effective Hamiltonian formalisms used in previous interpretations of anisotropically immobilized spin label spectra. That the true order parameters may be the same can be shown by including slow motional corrections in the effective Hamiltonian formalism. The larger volume of the doxyl ring may change the apparent order parameter by increasing the importance of the slow motional effects, as opposed to causing a real decrease in the order parameter, as previously proposed.

Seelig & Niederberger (1974), Seelig & Seelig (1974), and Stockton et al. (1976) have found that bilayers have differences in the order parameter, S, as determined from deuterium magnetic resonance (^{2}H NMR) experiments with selectively deuterated hydrocarbon chains, as opposed to S as determined from spin-labeling experiments. They found that S determined by deuterium labeling, S^d, is generally greater than S determined from spin labeling, S^s. An example of the published data is shown in Table I. The stearic acid spin probes are thought to introduce distortion of the bilayer at the site of the doxyl ring, and to reflect the response of the bilayer to the probe perturbation. The nitroxide disruption of the bilayer is reflected in this view by a generally lower order parameter resulting in $S^s < S^d$ (Seelig & Niederberger, 1974; Stockton et al., 1976; Seelig, 1977).

McFarland & McConnell (1971) have proposed that a cooperative tilt of the phospholipid chain near the polar head group could account for the flexibility gradient observed in spin-labeling experiments. They have also noted that, if the lifetime of the statistically tilted chain regions were short on the deuterium resonance time scale (10^{-5} to 10^{-6} s) but long on the ESR time scale (10^{-7} to 10^{-8} s), S^d would show a lower gradient of order than S^s (Gaffney & McConnell, 1974). Seelig & Seelig (1974) have observed that in this case S^s would be greater than S^d. The egg yolk lecithin bilayer appears to be an example where a time-dependent tilting of the hydrocarbon chain is responsible for $S^s > S^d$ (Gaffney & McConnell, 1974; McConnell, 1976; Seelig & Seelig, 1977).

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