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Spin-Label Studies of the Sulfhydryl Environment in Bovine Plasma Albumin. 1. The N-F Transition and Acid Expansion†

Charles N. Cornell and Lawrence J. Kaplan*

ABSTRACT: The environment of the sulfhydryl group in plasma albumin was previously characterized by employing spin-labels of varying chain lengths (Hull, H. H., Chang, R., & Kaplan, L. J. (1975) *Biochim. Biophys. Acta 400,* 132). It was established that the sulfhydryl is in a crevice approximately 10 Å deep but this crevice was not identified further. We now report the results of titrating albumin through the acidic conformational transitions while monitoring the electron-spin resonance of the bound nitroxide. With short spin-labels a general change is observed as the pH is lowered but the N-F transition is not discernible. However, with a spin label previously shown to project to the lip of the crevice a clear N-F transition as well as the subsequent acid expansion are observed. These results indicate that the sulfhydryl is in the crevice, formed by the domains of albumin, which opens during the N-F transition. Further results indicate that bound fatty acids do not influence the integrity of the sulfhydryl environment at neutral pH.

Bovine plasma albumin is known to undergo several pH dependent conformational transitions. On the acidic side of the isoionic point BPA[1] undergoes the reversible structural isomerization known as the N-F transition (Aoki & Foster, 1956) and the so-called acid expansion (Yang & Foster, 1954). A large number of studies have been undertaken to elucidate the nature and details of these transitions (see Foster (1960) for a review of the early literature and Peters (1975) for a recent review). In general, these and subsequent studies (Sogami & Foster, 1968) have revealed that, as the pH is lowered, the protein undergoes a fairly cooperative conformational change (N-F transition) that involves a slight expansion of the molecule resulting from a separation of intramolecular domains and the opening of a crevice. Further decrease of pH causes the protein to undergo a more complete expansion with a substantial increase in flexibility of the molecular structure.

BPA contains 17 disulfide bonds and (in the mercaptalbumin fraction) a single reactive sulfhydryl group located relatively close to the amino-terminal end of the polypeptide chain (King & Spencer, 1972; Brown, 1975). While the sulfhydryl has long been implicated in important physiological functions (King, 1961; Eagle et al., 1960; Putnam, 1965), recent in vitro evidence has demonstrated the specific catalytic role the sulfhydryl plays in the formation of a disulfide interchanged isomer (Nikkel & Foster, 1971; Stroupe & Foster, 1973). This isomer is also formed in vivo and is an intermediate in the catabolism of albumin (Wallevik, 1976). Additional isomers are formed when the sulfhydryl is oxidized to oxidation states higher than the well-known mixed disulfides (Janatova et al., 1968).

Due to the importance of the sulfhydryl group, it appeared to us that a specific study of the environment of the sulfhydryl residue and the conformational changes that take place in the

† From Thompson Chemical Laboratory, Williams College, Williamstown, Massachusetts 01267. *Received October 3, 1977.* Supported by funds from a grant to Williams College by the Alfred P. Sloan Foundation.

[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; ESR, electron spin resonance; MSL I, II, III, IV, and V, the maleimide spin labels whose structures are shown in Figure 1.

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region of the sulfhydryl residue during the acidic and basic (Cornell & Kaplan, 1978) transitions would be valuable. Along these lines Noel & Hunter (1972) have concluded that the sulfhydryl is located in a crevice which opens during the N-F transition. Hull et al. (1975), employing sulfhydryl specific, spin-label probes and electron spin resonance spectroscopy, obtained results consistent with the crevice model and indicated a depth of approximately 10 Å. On the other hand, Ohkubo (1969) has reported that the sulfhydryl group in human serum albumin is located at the border between a polar helical segment and a hydrophobic area on the surface of the protein.

The ESR spin-labeling technique seemed like the method of choice to investigate the geometry of the specific site in the protein since a nitroxide radical, introduced through use of a particular attaching group, acts as a reporter group reflecting the rotational freedom of the environment in which it resides (McConnell & McFarland, 1970; Griffith & Waggoner, 1969).

In the present investigation a series of nitroxide spin labels with a maleimide attaching group (MSL) has been employed to monitor the conformational changes which take place in the environment of the sulfhydryl residue of BPA during the course of the N-F transition and acid expansion. In addition, the "molecular dipstick" technique (Hsia & Piette, 1969) was used to determine the topology of the sulfhydryl environment in the different conformers of albumin.

Experimental Section

Preparation of Charcoal-Treated Albumin. Bovine plasma albumin, fraction V, lot 74-C-0253 (Sigma Chemical Co.), was charcoal defatted according to the procedure of Chen (1967) as modified by Sogami & Foster (1968).

Blocking and Determination of Sulfhydryl Groups. The free sulfhydryl group was blocked by reaction with an equimolar amount of iodoacetamide in a 0.1 M sodium phosphate buffer, pH 7.0. The solution was then exhaustively dialyzed against excess buffer.

Free sulfhydryl content of bovine plasma albumin and the derivatives prepared as indicated above was determined by the Ellman procedure (1959) observing the precautions cited by Janatova et al. (1968).

The sulfhydryl titer for charcoal-treated BPA was 60%. After selective spin labeling at the sulfhydryl residue or blocking with iodoacetamide, the titer was essentially zero.

Spin Labeling the Sulfhydryl or Amino Group. The nitroxide spin labels containing the maleimide attaching group were obtained from Syva Associates. Their structures and chain lengths are shown in Figure 1. To label the sulfhydryl group, a 10% solution of charcoal-treated albumin in a 0.1 M phosphate buffer (pH 6.4) was used while labeling of the amino group was accomplished with charcoal-treated, iodoacetamide blocked albumin in a 0.1 M phosphate buffer (pH 7.8). In each case, an equimolar amount of spin label was employed and the reaction was allowed to proceed with stirring for 24 h at 4 °C. The solution was then exhaustively dialyzed to remove unbound spin label against 0.1 M phosphate buffer, pH 6.4, when the series of spin labels was used or against 0.1 M NaCl in preparation for titration.

Titration of Spin-Labeled Albumin. A stock albumin solution was titrated using a Beckman Century SS-1 pH meter equipped with a Radiometer combination electrode (no. GK2321C). As the pH was lowered, 200-μL aliquots were removed from the stock solution and placed in an ESR tube. Fairly concentrated hydrochloric acid was used to minimize dilution and the other conditions were chosen to duplicate those of Sogami & Foster (1968).

Determination of ESR Spectra. The albumin solutions were placed in 4-mm Pyrex capillary tubing sealed at one end. The ESR spectra were recorded at ambient temperature in a Varian E-3 spectrometer. The ESR spectra were obtained within 2 h of the pH adjustment.

Miscellaneous. Protein concentrations were determined by absorbance measurements in a Gilford 240 spectrophotometer. The extinction coefficient of BPA was assumed to be $E_{1cm}^{1\%} = 6.67$ at 279 nm. The molecular weight of albumin was assumed to be 67 000 for use in the calculation of sulfhydryl titer.

Dialysis tubing with a molecular weight cutoff of 12 000 was obtained from A. H. Thomas. It was pretreated by repeated boiling in half-saturated $NaHCO_3$ followed by extensive washing with distilled-deionized water.

Deionized water with a specific resistance of greater than 10^6 ohms cm was obtained by passing distilled water through a mixed bed ion-exchange column (Bantam Model BD-1, Barnstead Still and Sterilizer Co.).

Results and Discussion

Spin-Labeled Sulfhydryl Residue in Charcoal-Treated BPA. Since albumin is one of the chief vehicles for transport of small lipophilic molecules and since Noel & Hunter (1972) indicated that one of the primary fatty acid binding sites is located adjacent to the sulfhydryl containing crevice, it was essential to determine if the molecular dipstick results were effected by fatty acid. When the maleimide spin labels I-V were bound to the sulfhydryl group of charcoal-treated BPA at neutral pH, spectra as shown in Figure 1a were obtained. The rotational correlation times, τ_c, were calculated from these spectra and a graph of τ_c vs. chain length (Figure 2) was constructed. The τ_c values were calculated according to the procedure described by Smith (1972) using the immobilized contribution of the spectra of spin labels I, II, III and the spectra of spin-labels IV and V as indicated by Hull et al. (1975). Even though the calculation is not strictly valid for the relatively immobilized spin labels, the general trend of τ_c as a function of chain length can be obtained. From the graph one can see that a sharp break occurs at 9.5-10.0 Å. The sharp break indicates the emergence of the nitroxide group from a restricted environment to an environment where relatively free rotation can occur. The distance at which this break occurs has been interpreted as the minimum depth of the sulfhydryl-containing crevice (Hsia & Piette, 1969; Chignell & Starkweather, 1973).

These data agree quite well with the data of Hull et al. (1975) for untreated albumin also shown in the figure and indicate that the fatty acid has little or no effect on the molecular dipstick results. However, Sogami & Foster (1968) have clearly shown that charcoal-treated albumin is more labile than nontreated albumin since the midpoint of the N-F transition is shifted to higher pH upon defatting. In addition, Andersson (1969) has shown that the lipophilic material aids in in vitro renaturation after denaturation of the albumin although the results of Steinhardt & co-workers (1972) indicated that fatty acid renatured albumin is not fully restored to its native state. Therefore, while the fatty acids undoubtedly stabilize the overall three-dimensional structure, our results indicate that they do not affect the integrity of the sulfhydryl environment at neutral pH.

N-F Transition of Sulfhydryl Spin-Labeled Albumin. From their study of spin-labeled albumin, Hull et al. (1975) concluded that the sulfhydryl was in a crevice 10 Å deep but were unable to further characterize the crevice. In an attempt to verify the contention of Noel & Hunter (1972) that a sulf-

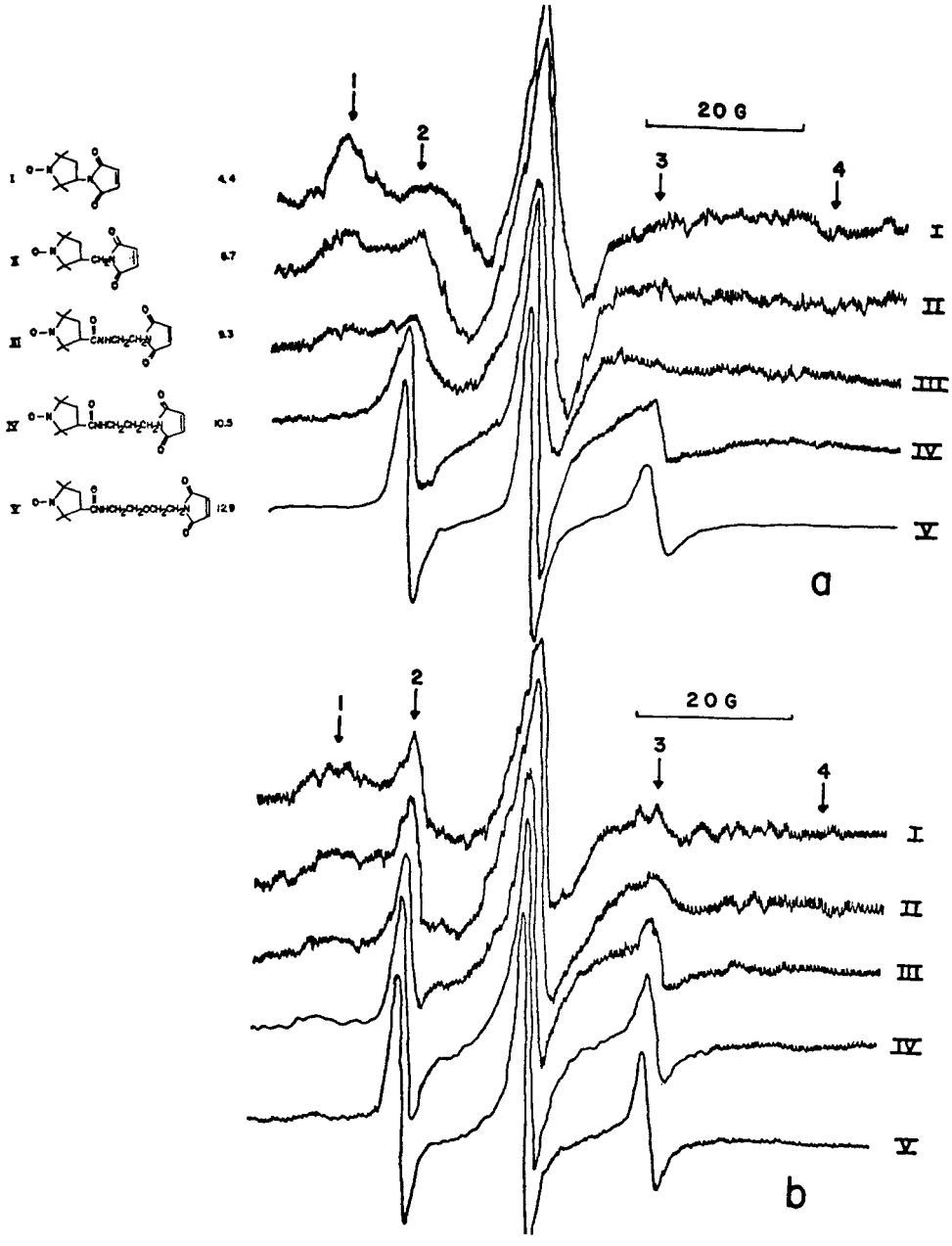

FIGURE 1: ESR spectra of maleimide spin-labeled albumin. Arrows 1 and 4 indicate the location of the low- and high-field peaks that are characteristic of a highly immobilized nitroxide radical. Arrows 2 and 3 indicate the analogous peaks for a freely rotating radical. (a) Albumin spin labeled on sulfhydryl group. (b) Albumin spin labeled on amino group after blocking the free sulfhydryl group.

hydryl-containing crevice opens in the N-F transition, we titrated albumin containing various spin labels through the acid conformational transitions. With the shortest spin-label MSL I bound to the sulfhydryl, little change in the spectra was observed as BPA was titrated through the N-F transition assumed to occur about pH 4.5-4.0 (Figure 3a). This is essentially the same result obtained by Stone et al. (1965) and Benga & Strach (1975) using a very short spin label and suggests that the environment of this spin label does not change appreciably during the N-F transition. Since all these investigations were performed with very short spin labels, we reasoned that the nitroxide was held so close to the molecular structure that it simply was not sensitive to the structural changes taking place during the N-F transition. This was clearly the situation.

When albumin, labeled with a spin label of a length such that it projects almost to the lip of the sulfhydryl crevice (MSL III), was titrated through the N-F transition, a substantial change in the ESR spectra was noted (Figure 3b). The rotational correlation times for the three shortest spin labels, MSL I, II, and III, as a function of pH, are shown in Figure 4. The calculation of τ_c was made using the dominant high field and low-field peaks as well as the center peak at each pH. An increase in the freedom of the nitroxide radical for MSL II and III is quite evident and it is clear that during the course of the N-F transition, the environment of the spin labels attached to the sulfhydryl undergoes substantial change.

From specific rotation and solubility data, Sogami & Foster (1968) reported the midpoints of the N-F transition to be pH 4.15 and 4.33, respectively. The fact that the midpoints of the curves reported here are shifted to a higher pH may be the result of a perturbing or destabilizing effect caused by the spin label. All of these data provide support for the contention of Noel & Hunter (1972) that the sulfhydryl is in a crevice which opens during the N-F transition.

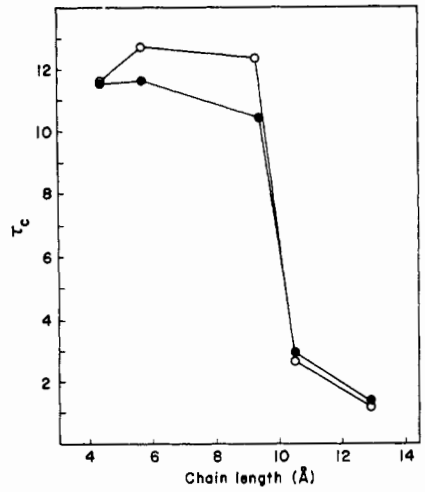

FIGURE 2: Rotational correlation in nanoseconds vs. chain length of the spin labels. (Open circles) untreated albumin which corresponds to the data of Hull et al. (1975) but calculated employing a different baseline; (filled circles) charcoal-treated albumin.

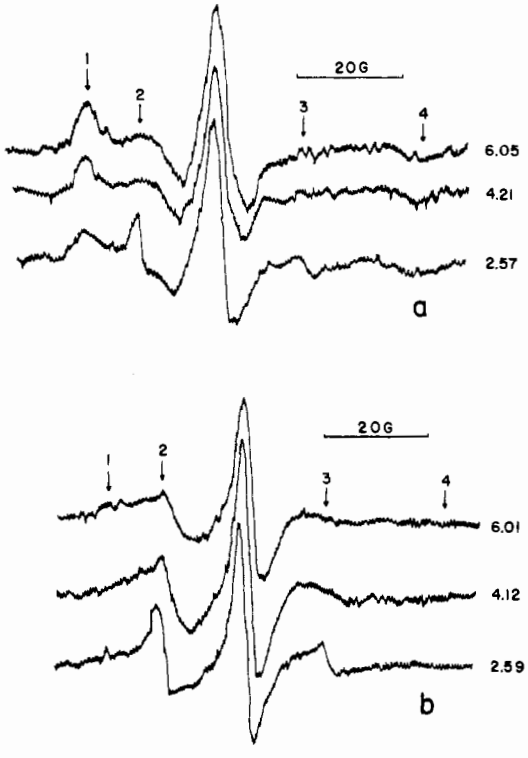

FIGURE 3: ESR spectra of spin-labeled BPA at pH values corresponding to the various conformers. (a) Albumin labeled with MSL I at pH 6.05, N form; pH 4.21, F form; and pH 2.57, acid expanded form. (b) Albumin labeled with MSL III at pH 6.06, N form; pH 4.12, F form; and pH 2.59, acid-expanded form.

Acid Expansion of Sulfhydryl Spin-Labeled Albumin. If the pH is lowered still further, the albumin molecule undergoes the acid expansion. The course of this reaction also was monitored by ESR with the three spin labels (MSL I, II, and III) attached to the sulfhydryl residue. A comparison of the spectra obtained with I and III is shown in Figure 3. As albumin undergoes the acid expansion, it was possible to observe a decrease in τ_c with even the shortest spin label, MSL I (Figure 4), which indicates a further rotational freedom of the spin label and therefore implies a more open crevice or unfolded molecule in the expanded form (as compared with the F form). This undoubtedly was the conformational change observed by

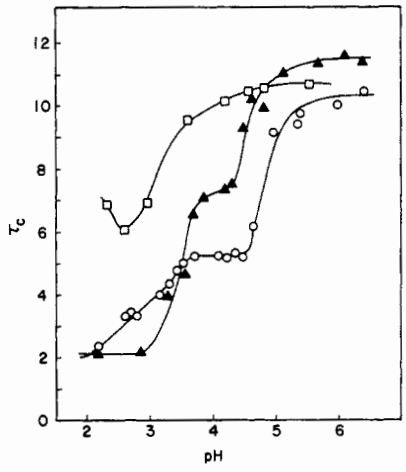

FIGURE 4: Rotational correlation times for albumin spin labeled on the sulfhydryl group during titration through the N–F transition and acid expansion. Albumin labeled with MSL I (squares), MSL II (filled triangles), and MSL III (circles).

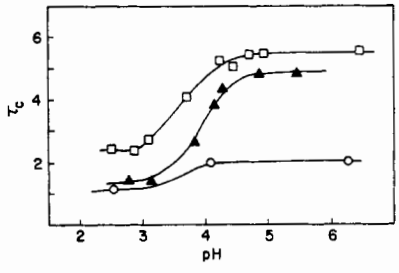

FIGURE 5: Rotational correlation times for albumin spin labeled on an amino group during titration through the N–F transition and acid expansion. Albumin labeled with MSL I (squares), MSL II (filled triangles), and MSL III (circles).

Stone et al. (1965) and Benga & Strach (1975) when they reported a major change in the ESR spectra as the pH was lowered from 5.5 to 2.5.

Spin-Labeled Amino Residue in Charcoal-Treated BPA. In order to determine the sensitivity of the spin label for its environment, we decided to compare the spectra of the sulfhydryl bound spin labels with those bound to an amino residue, presumably on the surface of the molecule. A greater freedom of rotation is evident (Figure 1b) for all the spin labels attached to an amino residue. It is interesting to note, however, that the spectra of MSL I and II contain a considerable contribution of a relatively immobilized nitroxide radical. While they exhibited relatively more freedom than the sulfhydryl bound nitroxide, not even the longest spin label displayed the highly mobile spectrum reported for the short spin label bound to sulfhydryl known to be on the surface of carbonic anhydrase (Erlich et al., 1973). τ_c values were calculated from these spectra using the peaks associated with the nitroxide in an unrestricted environment.

N–F Transition and Acid Expansion of Albumin Spin-Labeled at an Amino Residue. Since the spin-labeling results indicated that the sulfhydryl residue was in a crevice and that its environment becomes less restrictive as albumin undergoes the acid transitions, we selectively labeled an amino group, to compare its relative environment with that of the sulfhydryl residue as albumin undergoes the acid transitions. As expected, the environment of the spin label attached to an amino group of iodoacetamide-blocked albumin changed relatively little as the protein was titrated through the acid transitions (Figure 5).

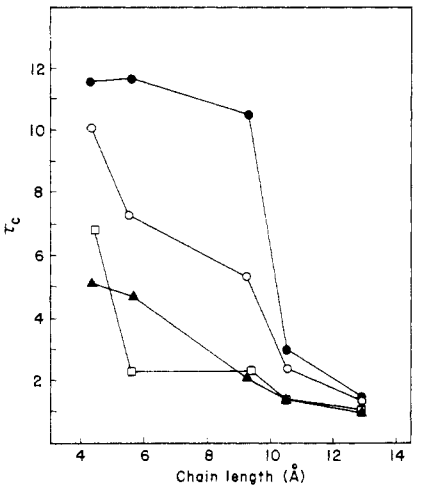

FIGURE 6: Rotational correlation times vs. chain length of the spin labels for the different albumin conformers labeled on the sulfhydryl group and for albumin labeled on the amino group. (Filled circles) N; (open circles) F; (open squares) acid expanded; (filled triangles) albumin labeled on amino group.

Further Discussion. In order to characterize comparatively the free sulfhydryl environment in the N, F, and acid-expanded forms, τ_c was plotted against spin-label chain length for these conformers of BPA (Figure 6). Also for comparison τ_c for the spin labels bound to the amino group are included. The sharp break in the curve for native BPA indicates that the sulfhydryl is in a restrictive, narrow crevice. In the F isomer, this crevice opens changing the sulfhydryl environment. A gradual change in τ_c, as observed in this case, has been interpreted by Chignell and co-workers (1972) to indicate a funnel-shaped crevice. This would be consistent with what is known about the mechanism of the N–F transition but may not be strictly correct since the spin label bound to an amino group also exhibits a linear τ_c vs. chain length curve. (Further details of the environment of the amino group are considered in a study of human plasma albumin (Cornell, C. N., Chang, R., & Kaplan, L. J., submitted).) An interpretation consistent with both sets of data is simply that the spin label is gradually escaping from restricting (nonspecific) interactions with the rest of the molecule. In the acid-expanded isomer, the sulfhydryl group is in a largely exposed environment (very similar to the amino-bound label) indicating a significant unfolding and loosening of the molecular structure resulting in an increased flexibility under these conditions. It should be pointed out that the disulfide bonds remain intact during these transitions and therefore the albumin never approaches a complete random coil. Therefore, even in the state of greatest flexibility, the spin label will not approach the freedom of free solution since it is covalently bound to the huge, slowly tumbling albumin molecule.

Acknowledgments

We thank Dr. Raymond Chang for continued interest in this investigation and for helpful discussions.

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