The Neutral Transition and the Environment of the Sulfhydryl Side Chain of Bovine Plasma Albumin†

Vincent R. Zurawski, Jr., and Joseph F. Foster*

ABSTRACT: Using the reagent 3-bromo-1,1,1-trifluoropropanone, the free sulfhydryl residue of the mercaptalbumin fraction of bovine plasma albumin was modified to give the trifluoroacetonylated protein. Nuclear magnetic resonance (nmr) spectra of the F label were obtained as a function of pH and temperature using a Varian XL-100 spectrometer. At 37° in 0.16 M NaCl the singlet resonance peak corresponding to the N form of the protein (pH 5–6) was 375 Hz upfield from trifluoroacetic acid, while for the B form (pH 9–10) it was 334 Hz upfield. At intermediate pH, the magnitude of the chemical shift paralleled the relative proportion of N and B forms as judged by optical rotation at 300 nm. A very similar shift of the peak to 342 Hz upfield occurs in the conversion to the F form at pH 3.5–3.9. The pronounced effect of calcium ions on the neutral transition was also confirmed by both nmr and optical rotation methods. At ratios of calcium to albumin approximating that in plasma, the transition occurs close to physiological pH and the cooperativity in hydrogen ions is enhanced, Hill coefficients being 2–3 as compared to 1.0 in absence of calcium. On the basis of these results plus previous inferences about the character of the N–F and the N–B transitions, a possible model is proposed. In brief, it is suggested that electrostatic pairing of carboxylate and cationic sites in the N form of the protein bring the cysteine residue into close proximity with one or more imidazolium or other cationic residues and that these interactions are broken in either of the two transitions.

Both human and bovine plasma albumins undergo a pH-dependent conformation change in neutral or slightly alkaline solution (Leonard *et al.*, 1963). This transition has been designated the "neutral transition" (Harmsen *et al.*, 1971) to distinguish it from the better-known acid transitions which these proteins undergo (Yang and Foster, 1955; Aoki and Foster, 1957). Harmsen and coworkers (Harmsen *et al.*, 1971) have made the important observation that in the presence of calcium ion the midpoint of this transition is shifted downward close to physiological pH and the hydrogen ion cooperativity is enhanced. These results clearly suggest a linkage between calcium and hydrogen ion binding, and Harmsen and coworkers called attention to the analogy with the well-known oxygen Bohr effect in hemoglobin. Obviously, as a result of these new observations the neutral transition takes on great potential interest with respect to the biological role of the albumins.

The majority of the albumin molecules (mercaptalbumin fraction) contain a single reactive sulfhydryl group located relatively close to the amino-terminal end of the peptide chain (King and Spencer, 1970). Work in our laboratory (Nikkel and Foster, 1971; Stroupe and Foster, 1973) has demonstrated the ability of this functional group to catalyze a strictly intramolecular and reversible structural change in the protein which we believe to be a rearrangement of two or more disulfide bonds. The pH dependence of the rates of both the forward and reverse transformations probably reflects primarily the state of ionization of the sulfhydryl group. However, Stroupe and Foster (1973) pointed out that the pH dependence of the equilibrium constant for isomerization probably cannot be explained on this basis but most likely reflects the pH dependence of the neutral transition. This raised in our minds general questions about the possible relation of sulfhydryl ionization to the neutral transition and, conversely, possible alterations in the environment of the sulfhydryl residue accompanying the neutral transition.

Huestis and Raftery (1972) have succeeded in introducing a fluorinated probe into the β chain of hemoglobin at cysteine-93. Using ^{19}F nuclear magnetic resonance (nmr) spectroscopy they obtained information concerning conformational changes in hemoglobin associated with alterations in pH and binding of several ligands. The same approach has been utilized in the present paper to explore the environment of the sulfhydryl group in bovine plasma albumin and particularly to follow alterations in this environment in the neutral transition. Supporting evidence for the cooperative character of the transition has been obtained and the dramatic effect of calcium ions on the transition has been confirmed.

Experimental Section

Materials. Fraction V, lot G36612, and crystallized bovine plasma albumin, lots C78012 and F71601, were purchased from the Armour Pharmaceutical Co. 5,5′-Dithiobis(2-nitrobenzoic acid) was purchased from the Aldrich Chemical Co., Inc. 1-Butanol was purchased from the J. T. Baker Chemical Co. and distilled at 116–117° before use. Deuterium oxide (99.7% D_2O) was purchased from Columbia Organic Chemicals Co. NaOD was prepared by dissolving pure sodium metal in the D_2O. 3-Bromo-1,1,1-trifluoropropanone was obtained from Penninsular Chem Research, Inc., lot 2195. All other chemicals were of the highest quality commercially available.

All water was distilled and deionized with a specific resistance greater than 10^6 ohms, and was filtered through sintered glass before use. Dialysis tubing obtained from Union Carbide Corp. was boiled several times in deionized water and stored under water at 2° until used.

Albumin Preparations. Bovine plasma albumin (Armour Pharmaceutical Co.) either crystallized or fraction V, was defatted according to the procedure of Chen (1967), as modified in this laboratory (Sogami and Foster, 1968). Monomeric pro-

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tein was prepared by passage of the defatted material over a Sephadex G-150 column equilibrated with 0.16 M NaCl. Bovine mercaptalbumin was prepared according to the method of Hagenmaier and Foster (1971), using either SE- or SP-Sephadex. It should be noted that the SP-Sephadex is less satisfactory for this preparation, due to the slower flow rates it yields at the pH of the chromatographic separation, 4.30.

Protein solutions were filtered through a 0.22-μ Millipore filter and stored at pH 5.5 and 2° with a minimum of 0.15 M salt present, for no longer than 3 weeks.

Preparation of Trifluoroacetonylated Proteins. To a 10–12% solution of fraction V albumin in 0.2 M phosphate buffer at pH 7.2 was added the reagent 3-bromo-1,1,1-trifluoropropanone in sufficient amounts to give a molar ratio of 10:1 based on free sulfhydryl groups. The reagent was added volumetrically with constant, slow stirring.

The reaction was allowed to continue for 40–50 min with constant monitoring of pH to ensure complete modification, although alterations in pH appeared only in the first 15 min. No precipitate was produced as noticed in the case of hemoglobin (Huestis and Raftery, 1972). At the conclusion of the reaction the material was desalted by passage through a Sephadex G-25 column after adjustment of the pH to 5.5. The pooled and concentrated protein was then dialyzed *vs.* the appropriate final salt solution. In each case analysis for free sulfhydryl groups was used to check for completeness of reaction. Sometimes the material was lyophilized after dialysis *vs.* water. No detectable differences in properties were found upon redissolving.

Preparation of Trifluoroacetonylated Model Compound. S-Trifluoroacetonylmercaptoethanol was prepared using the direct combination method described by Huestis and Raftery (1972). Small quantities of reagents were generally used due to the exothermic nature of the reaction. The white crystalline product was washed with hexane, dissolved in 95% ethanol, recrystallized at 0°, and again washed with chilled hexane. Microanalysis verified the composition but showed it to be monohydrate (*Anal.* Calcd: C, 29.13; H, 4.40; F, 27.64; S, 15.55. Found: C, 29.02; H, 4.30; F, 27.60; S, 15.50).

Huestis and Raftery (1972) reported characterization of their product by infrared and proton nmr spectra. In an aqueous environment, however, one must question the existence of a carbonyl group in a molecule where hydration to the gem diol could be stabilized by electron-withdrawing fluorines. Indeed, in carbon tetrachloride, infrared spectra of this compound which had been exposed to water showed no characteristic carbonyl stretching peak in the vicinity of 1700 cm^{-1}, but did show at least two hydroxyl stretching peaks in the 3600-cm^{-1} region.

Optical Rotation Measurements. Optical rotatory dispersion measurements and optical rotation at 300 and 233 nm were carried out with a Cary 60 recording spectropolarimeter. The instrument was programmed to give a constant spectral bandwidth of 15 Å. Jacketed cells of 1- and 10-mm path length were used at 25 or 37 ± 0.1°. These cells were held firmly in place with a specially made device, screw tightened to the mounting block. The pH of a portion of the solution being examined was determined at the same time spectral measurements were being performed. Absolute values of specific rotation in the plateau pH regions are the averages of several experiments.

Solutions of identical protein concentration were prepared by addition of a stock solution, which had been dialyzed against the appropriate solvent, to a calibrated volumetric flask. In cases where pH was adjusted, acid and base solutions employed, usually 0.05 M, were made up to the appropriate ionic strength of 0.16 with sodium chloride. When calcium ions were present, all solutions contained the same concentrations. All solutions were filtered, protein solutions through rinsed 0.22-μ Millipore filters, others through fine sintered glass.

Moffitt–Yang parameters a_0 and b_0 (Moffitt and Yang, 1956) were statistically determined with dispersion data collected in the 500–300-nm wavelength range assuming λ_0 to be 218 nm (Sogami *et al.*, 1963). Appropriate corrections for dispersion of solvent refractive index were made. Comparison of normal Moffitt–Yang plots to plots where slope and intercept were inverted was useful in assessing reliability of the data.

Nmr Measurements. ^{19}F nmr spectra of the trifluoroacetonylated derivatives were recorded using a Varian XL-100 spectrometer operating at 94.077 MHz. The spectrometer was supplemented by a Fabritek Model 1080 computer of average transients, required for the accumulation of protein spectra. Sample tubes were 12 mm o.d. Resonance positions were measured at the indicated temperature from the H_2O proton lock signal and referenced to a standard trifluoroacetic acid resonance peak, determined at ambient temperature. This reference was the same as utilized by Huestis and Raftery (1972). In cases where solutions were made with D_2O or butanol, appropriate deuterium or proton locks were achieved; the trifluoroacetic acid peak was still used as a reference.

Protein solutions used for measurement contained approximately 25 g of protein/100 ml. Data for *S*-trifluoroacetonylmercaptoethanol solutions were obtained both at 10^{-2} and 10^{-3} M in each case. All protein spectra were obtained using 0.16 M NaCl as the solvent, or solutions with the designated calcium ion concentration and made up to ionic strength 0.16 with sodium chloride. In the cases where D_2O was used as a medium, NaOD was used to adjust the pD.

Miscellaneous. Protein concentrations were measured by scanning solutions with either a Cary 15 or a Cary 118 recording spectrophotometer assuming an extinction coefficient $E_{279}(1\%) = 6.67$. As a first approximation correction for any light scattering, final optical densities at 279 nm were determined by subtracting absorbance values at 350 nm from those at 279 nm. The values at 350 nm were always less than 0.010.

Free sulfhydryl content of bovine plasma albumin and derivatives was determined by the method of Ellman (1959), observing precautions necessitated by the protein concentration dependence observed by Janatova *et al.* (1968). A Tris-HCl buffer was employed to adjust the pH to 8.0–8.1. Solutions of 5,5′-dithiobis(2-nitrobenzoic acid) were prepared immediately prior to use.

Measurement of pH was performed using either a Radiometer 25 pH meter equipped with an expanded scale and a GK2302C combination electrode, or a Beckman Research pH meter equipped with a Beckman glass electrode (41263) and a Beckman calomel reference electrode (39071). Standardization and scale linearization were performed with Sargent standard buffers at values of pH 4, 7, and 10. It is estimated that reproducibility over extended periods was about ± 0.02 pH unit. The pD in D_2O solutions was assumed to be equal to the indicated pH plus 0.4 (Lumry *et al.*, 1951).

Results

Figure 1 shows the neutral transition as monitored by specific rotation at 300 nm. Similar transition curves were carried out on defatted preparations of whole crystallized albumin, both with and without the small amount of dimer removed, and on a highly purified sample of mercaptalbumin monomer (Hagenmaier and Foster, 1971). In all cases the results were the

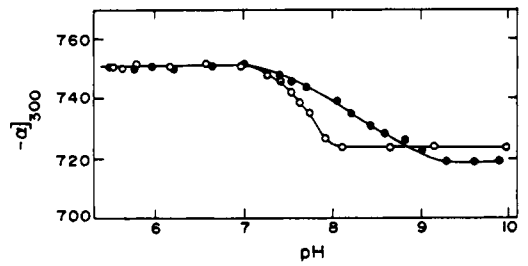

FIGURE 1: $-\alpha]_{300}$ *vs.* pH for bovine plasma albumin in the neutral transition region. Data are for 1.3% bovine plasma albumin in 0.16 M NaCl (●) and in 0.154 M NaCl–0.002 M $CaCl_2$ (○) at 25°. Without calcium ions, the midpoint pH is 8.36, the Hill coefficient, 1.00; with calcium ions, the midpoint pH is 7.68, the Hill coefficient, 2.69.

same within experimental error. Figure 1 shows results in two supporting electrolytes, 0.16 M NaCl and a solution of the same ionic strength but containing 0.002 M $CaCl_2$. This latter system was chosen to approximate normal plasma. The resulting curves are in excellent agreement with those of Leonard and coworkers (Leonard *et al.*, 1963) and Harmsen and coworkers (Harmsen *et al.*, 1971). In particular they demonstrate the marked effect of calcium ions. In the case cited the midpoint of the transition is shifted downward from 8.36 to 7.68 and the Hill coefficient increased from 1.00 to 2.69.

A small decrease in the magnitude of the rotation at the 233-nm trough was also observed in the neutral transition. The value of $-\alpha]_{233}$ was found to be 9.1×10^3 deg for the N form and 8.5×10^3 deg for B. This result, in substantial agreement with corresponding data presented by Wallevik (1973) for human albumin, suggests a small but measurable decrease in helix content on conversion of N[1] to B which is also supported by the small decrease in the b_0 dispersion parameter (Table I). Interestingly, this apparent decrease in helix content is almost totally eliminated in the presence of calcium ions as seen by the constancy of b_0 (Table II) and the fact that $-\alpha]_{233}$ decreased only from 9.1×10^3 deg to 8.9×10^3 deg in presence of calcium. In this case the transition appears to behave purely as a tertiary conformation change as suggested by Leonard *et al.* (1963).

Introduction of a ^{19}F probe at the free sulfhydryl residue was accomplished by reaction of bovine plasma albumin with the reagent 3-bromo-1,1,1-trifluoropropanone as outlined in the Methods section. That derivatization took place specifically on the sulfhydryl group was demonstrated by the absence of any ^{19}F signal in a control preparation in which the derivatization reaction was carried out after first blocking the sulfhydryl residue by reaction with iodoacetamide. This later reagent is known to react exclusively and quantitatively with the sulfhydryl function in bovine plasma albumin under the conditions employed (Nikkel and Foster, 1971). The protein labeled with the ^{19}F marker can also be considered to exhibit native behavior, at least with respect to the neutral transition. This is demonstrated by the data presented in Figure 2 showing the pH dependence of the specific rotation to be indistinguishable in labeled and unlabeled proteins.

In all cases the ^{19}F signal from the trifluoroacetonylated derivative of albumin was seen as a single broad resonance peak. A typical example is given in Figure 3, in this case at pH 5.5 and 37°. Table III gives the chemical shifts of the singlet peak,

TABLE I: Values of a_0 and b_0 for Bovine Plasma Albumin in 0.16 M NaCl, at Various Values of pH, $T = 25°$.

pH	$-a_0$	$-b_0$	Standard Error
5.65	329	256	±1.8
6.25	326	260	±2.9
7.64	333	251	±2.2
8.27	320	248	±1.9
8.89	312	246	±2.1
9.65	311	247	±2.5
9.99	310	249	±2.1

TABLE II: Values of a_0 and b_0 for Bovine Plasma Albumin in 0.154 M NaCl–0.002 M $CaCl_2$ at Various Values of pH, $T = 25°$.

pH	$-a_0$	$-b_0$	Standard Error
6.38	330	260	±3.1
7.06	330	260	±4.9
7.55	326	255	±3.9
8.04	302	263	±3.9
8.85	303	258	±6.1
9.62	302	266	±2.8

measured upfield from a reference trifluoroacetic acid peak, for various temperatures and pH values in 0.16 M NaCl. At low pH, problems arose from the gelation of highly concentrated protein solutions making determinations above 32° impossible. These data show similar chemical shifts in the F and B conformational states while in the acid-expanded region quite a different value is found. The resonance peak of the acid-expander conformer is also considerably broadened relative to the others. These results are a good indication that the chemical shift values are dependent on the structural states of the protein rather than solely on the titration of groups in the vicinity of the probe. This is also supported by the coincidence of the transition curves illustrated in Figure 4. In order to facilitate comparison of the chemical shift data with the transition as observed by optical rotation, the fractional change in the property has been calculated in both cases and plotted as fraction of the N form *vs.* pH.

Using approximately physiological ratios of calcium ions to ^{19}F-labeled albumin, chemical shift *vs.* pH curves were ob-

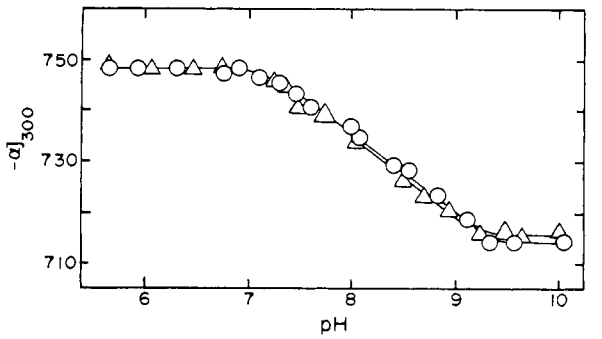

FIGURE 2: $-\alpha]_{300}$ *vs.* pH for bovine plasma albumin (△) and the trifluoroacetonyl derivative of albumin (○) in 0.16 M NaCl, $T = 25°$. Protein concentration was 0.13% in each case. For the trifluoroacetonyl derivative the midpoint pH is 8.33, the Hill coefficient, 1.00.

[1] Abbreviations used are: N, the normal conformer of bovine plasma albumin existing in the isoionic pH range; B, the basic conformer arising in the neutral transition; F, the conformer existing in the pH 3.5–4.0 range.

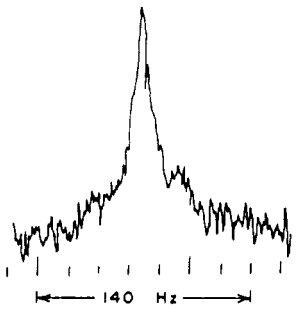

FIGURE 3: A typical ^{19}F resonance peak (400 scans) for the trifluoroacetonyl derivative of bovine plasma albumin obtained at pH 5.5, 37°, 375 Hz upfield of trifluoroacetic acid.

tained (see Figure 5). The same shifting and sharpening of the curve noted in the specific rotation measurements is evident, adding further support to the conclusion that the observed changes in chemical shift are functions of the conformational changes involved in the N-B transition. The difference in the plateau levels in the B form of the protein with and without calcium present is not entirely unexpected, since small differences in $[\alpha]_{300}$ plateaus were also noted. When calcium ions are present there is also a downfield shift of the signal near pH 9.5, which is not seen in the absence of calcium. These effects of calcium ion cannot be attributed to any direct interaction with the ^{19}F probe, in view of the results presented in Table IV showing that calcium has no effect at all on the chemical shift exhibited by the model compound, *S*-trifluoroacetonylmercaptoethanol.

Table IV includes additional values of the chemical shift of both the model compound and the protein under a variety of conditions. To a first approximation it appears that solvent exposure of the probe in the N and B conformers must be the same since each yields about a 25-Hz shift in the presence of D_2O, comparable to the 31-Hz shift seen in the model compound. Evidently the alteration in the environment of the sulfhydryl residue in the neutral transition cannot be represented in terms of a simple change in exposure to solvent. This is further supported by the very large upfield shift of the model compound in butanol where the probe is clearly in an environment of lower dielectric constant, *i.e.*, a more hydrophobic environment, indicating that a true buried-to-exposed model would probably require a substantially larger shift than the 41 Hz observed. The acid expansion may involve such a change: note the large upfield shift in going from pH 5 to 2 (Table III).

TABLE III: ^{19}F Chemical Shift Values for Various Conformers of the Trifluoroacetonyl Derivative of Bovine Plasma Albumin in 0.16 M NaCl.

	Chemical Shift[a] at Temperature (°C)				
pH (Conformer)	19	25	32	35	37[b]
9.50 (B)					334
5.58 (N)	412	394	384	374	375
3.70 (F)		362	350		(342)
2.09 (acid expanded)		501	489		(480)

[a] Hz upfield of trifluoroacetic acid (error estimated ±2 Hz). [b] Figures in parentheses are calculated values assuming a linear relation of chemical shift *vs.* temperature as found in the N form of the protein.

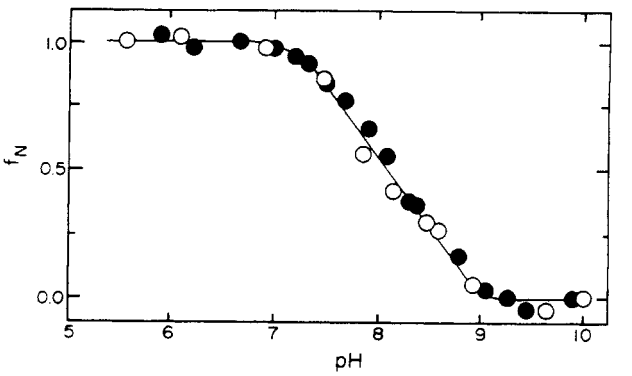

FIGURE 4: Fraction N *vs.* pH using normalized data obtained from $-\alpha]_{300}$ (●); and from ^{19}F chemical shift (○). The midpoint pH for the curve is 8.17, the Hill coefficient is 1.00.

Discussion

It is most significant that the specific rotation parameter $-\alpha]_{300}$ and the chemical shift of the ^{19}F label show precisely the same pH dependence within experimental error (Figure 4). Since the one parameter presumably represents a resultant of conformational changes in the peptide backbone or other general conformational changes while the nmr parameter reflects a strictly localized environmental alteration close to the sulfhydryl locus, this result strongly supports the contention that the neutral transition may be regarded as two state. While the existence of intermediates certainly cannot be ruled out by such an argument, it does seem clear that they cannot make an important contribution in quantitative terms. To the extent this is a valid conclusion the reaction may be expressed simply as

$$N \rightleftharpoons B + qH^+ \quad (1)$$

and the coefficient q at the midpoint of the transition would be given by the observed Hill coefficient. In the absence of calcium ions this coefficient was invariably found to be 1.0 within experimental error implying only a single hydrogen ion to be released in the transition.[2]

Harmsen *et al.* (1971) made the tacit assumption that the transition is two state and proposed that it involves an unmasking of imidazolium residues. Specifically, they showed that the hydrogen ion titration curve through the region of the neutral transition can be fitted assuming 10 masked imidazolium residues in N to be normalized when the protein is converted to the B form. Unfortunately, in view of the observed Hill coefficient, this attractive model is in conflict with the proposition that the transition is two state. The model of Harmsen and coworkers would demand a difference in protonation between N and B of approximately 5-10, increasing as the pH is raised through the range of the transition. It follows that if the two-state model prevails, the coefficient q must also be approximately 5-10, totally at odds with the observed Hill coefficient of 1.0. It must be concluded that either (1) the reaction is not two state and the "cooperative unit" is much smaller than the total molecule, or (2) the model of Harmsen and coworkers is incorrect and the difference in hydrogen ion binding between N and B is much smaller than their model would predict, indeed approximately 1.0. The fact that the presence of calcium ions resulted in a significant increase in the Hill coefficient from 1.0 to 2-3 might be taken as lending some support to the second alterna-

[2] This result has also been obtained for human albumin by Wallevik (1973) who showed furthermore a slight increase in the Hill coefficient with increasing temperature and noted the implication that the N-B equilibrium must not be strictly two state.

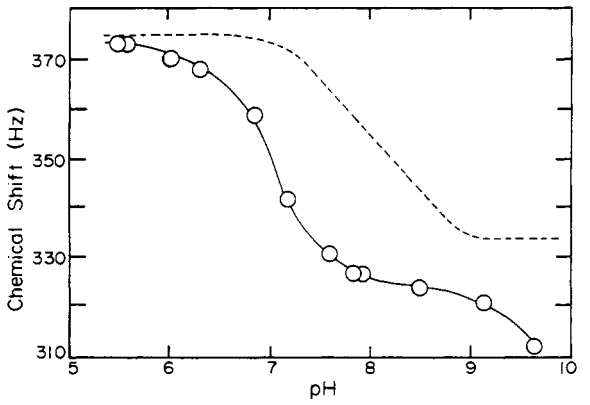

FIGURE 5: Chemical shift for the trifluoroacetonyl derivative of bovine plasma albumin in Hz upfield from trifluoroacetic acid *vs.* pH, in 0.100 M NaCl-0.02 M $CaCl_2$ at 37°. Dashed line represents curve with no calcium ions present.

tive. At a given concentration of calcium ions the B form clearly must bind more Ca^{2+} than does N (see below). Moreover, the binding of Ca^{2+} would almost certainly be accompanied by some release of bound hydrogen ions, particularly if binding followed a model similar to that proposed by Saroff and Lewis (1963). Consequently, in the presence of calcium ions it is to be expected that the difference in hydrogen ion binding between N and B, namely q in eq 1, would be increased.

The possibility should also be considered that microheterogeneity of the protein is responsible for the apparent discrepancy between the observed and expected Hill coefficients, in a manner similar to that in which we explained the corresponding discrepancy observed in the N-F transition (Foster *et al.*, 1965). In the event the protein consisted of a population of molecules undergoing transition at slightly differing pH the shape of the transition curve would have no thermodynamic significance but would reflect the distribution of molecular species. We have no evidence that microheterogeneity is a contributing factor in the neutral transition and indeed there are grounds that lead us to doubt that it is significant. In the first place we have never observed any evidence for electrophoretic resolution of N and B species, in contrast to the situation prevailing in the range of the N-F transition which was the principal factor forcing us to the microheterogeneity concept (Foster *et al.*, 1965). Secondly, as discussed later, the mercaptalbumin and nonmercaptalbumin components evidently exhibit the same pH dependence for the N-B transition and it is known that the nonmercaptalbumin fraction is much more microheterogeneous than is the mercaptalbumin (Kaplan and Foster, 1971).

Whereas ionization of the sulfhydryl group of mercaptalbumin must take place in a similar pH range as the neutral transition, it seems clear that this ionization is not directly involved in the transition. This can be readily inferred from the fact that derivatization of the sulfhydryl group with the trifluoroacetonyl label does not affect the pH dependence of the transition (Figure 2). It should also be pointed out that these experiments were performed with albumin, not mercaptalbumin, so that only approximately two-thirds of the protein molecules were labeled in the nmr experiments. It seems clear that the transition behavior of mercaptalbumin and nonmercaptalbumin components is indistinguishable, supporting the noninvolvement of the sulfhydryl residue. A corollary of this conclusion is that the pH dependence of the transition cannot be related to ionization of the sulfhydryl group, which must have substantially the same pK in both N and B conformations. Goldsack and Waern (1971) have suggested that the pK of the free sulfhydryl is different in the N and B states of the protein. They offer no direct evidence for a pK alteration of this particular group, however, Nevertheless, while the sulfhydryl group cannot be involved directly, the transition clearly results in some alteration in the local environment of this group which is reflected in a change in the chemical shift of the ^{19}F label.

TABLE IV: ^{19}F Chemical Shift Values for Trifluoroacetonyl Derivatives of Mercaptoethanol and Bovine Plasma Albumin at 37° in Various Solvents.

Sample	Solvent	Chemical Shift[a]
Trifluoroacetonyl-mercaptoethanol	0.16 M NaCl-H_2O	674[b]
Trifluoroacetonyl-mercaptoethanol	0.02 and 0.002 M $CaCl_2$ in NaCl-H_2O $\Gamma/2 = 0.16$	674[b]
Trifluoroacetonyl-mercaptoethanol	0.16 M NaCl-D_2O	705
Trifluoroacetonyl-mercaptoethanol	1-Butanol	821
Trifluoroacetonyl-albumin	0.16 M NaCl-D_2O pD 6.12	399
Trifluoroacetonyl-albumin	0.16 M NaCl-D_2O pD 10.05	361

[a] Hz upfield of trifluoroacetic acid (error estimated ±1 Hz in model compound; ±2 Hz for protein derivative). [b] Identical values were obtained from pH 2 through pH 8. At values of pH above 8 a slight decrease in chemical shift was noted in each case.

Since only a singlet is seen in all cases it can be inferred that the maximum lifetime of each of the conformational states is only of the order 10^{-3} sec or less. Goldsack and Waern (1971) assign relaxation rates of 100-400 msec to the neutral transition based on pressure jump studies. It seems, however, that in a system where very high pressures are utilized some other perturbation of the albumin molecule might be manifested by these slow relaxation times. Wallevik (1973) has concluded, for example, that the thermal unfolding and the N-B transition of human albumin are virtually independent processes in the neutral and slightly alkaline pH range. As indicated earlier, the fact that D_2O results in shifts in both the N and B plateau regions which are similar and comparable to those found with the model compound suggests the environment of the sulfhydryl residue to be essentially aqueous in both conformers. However, some caution must be taken in view of the observation that the model compound exists as the monohydrate. This raises the possibility that replacing H_2O by D_2O leads to an intramolecular rather than a general solvent effect, and that the environment of the sulfhydryl residue in the protein is not in fact comparable to that in free solution. Certainly, though, the solvent has ready access to the site in both states since the D_2O-shifted spectra, whether of an inter- or intramolecular origin or both, were seen in either case within a few minutes of dissolving the protein in the D_2O solvent.

It seems noteworthy that the shift in signal is virtually the same on going from the N form to either the F or B states. Leonard *et al.* (1963) called attention to the similarity of the neutral transition to the N-F transition. The present result at least emphasizes that the source of the shift cannot be the sim-

ple titration of a single prototropic group and further tempts us to seek a common explanation for the change in sulfhydryl environment in the two transitions. The possibility for a qualitative explanation is found in the work of Huestis and Raftery (1972) on hemoglobin together with what is already known about the essential character of the N-F and N-B transitions. There is considerable evidence that the N-F transition involves the rupture of a substantial number of ion-pair interactions between carboxylate groups and suitable cationic partners (Vijai and Foster, 1967). A similar explanation for the N-B transition has been given by Harmsen *et al.* (1971), in this case specifically invoking histidyl sites as cationic partners.

The present results are surprisingly similar to those of Huestis and Raftery (1972) on trifluoroacetonylated deoxyhemoglobin. They found the chemical shift corresponding to the ^{19}F label on $Cys^{\beta 93}$ to be 535 Hz upfield of the trifluoroacetic acid reference, while the oxy form yielded a value of 483 Hz, a 52 Hz difference. They also found that titration of the unoxygenated protein from approximately pH 7 to 8 gives a shift similar to that found in the neutral transition of albumin, approximately 40 Hz downfield. The oxyhemoglobin does not give such a shift in this range of pH, but maintains its base-line value of 483 Hz. Huestis and Raftery proposed that the signal change in each case was probably due to the removal of His^{146} of the β^- chain from its position forming a salt bridge with $Asp^{\beta 94}$, which is adjacent to the modified $Cys^{\beta 93}$. This would result in the loss of the imidazole ring current and/or charge influencing the chemical shift of the nearby probe. The removal of $His^{\beta 146}$ from its salt bridge with $Asp^{\beta 94}$ on oxygenation was identified by Perutz and coworkers from X-ray structures of the oxygenated and unoxygenated forms of hemoglobin (Perutz *et al.*, 1968; Perutz, 1970; Muirhead and Greer, 1970).

It is tempting to propose a very similar explanation for the observed alteration of the chemical shift of the ^{19}F label in the mercaptalbumin molecule. The amino acid sequence of bovine plasma albumin in the neighborhood of the free sulfhydryl group (King and Spencer, 1973) shows that there are two carboxylate side chains within four positions to the C-terminal side of the cysteine. Thus a portion of the polypeptide chain near the free sulfhydryl possesses a negative charge at neutral pH. There is also a cluster of hydrophobic and neutral residues immediately to the N-terminal side of this free sulfhydryl portion of the chain (King and Spencer, 1973). Furthermore, there is doubtless a reasonable amount of potential conformational freedom in this portion of the polypeptide chain since it is close to the amino terminus and not restricted by disulfide bonds. Thus, it is easy to imagine that in neutral solution the sulfhydryl group might be brought by electrostatic attraction close to cationic sites, possibly involving one or more histydyl residues. Either protonation of the carboxylate groups or deprotonation of the imidazolium groups, associated respectively with the N-F and N-B transitions, would result in movement of the free sulfhydryl away from these cationic groups and consequent loss of their perturbing effect on the ^{19}F label. Such movement would require no alteration of exposure to solvent.

Turning to the effect of calcium ions, it is clear, as shown by Harmsen *et al.* (1971) and the present results, that this divalent ion shifts the neutral transition toward the B state and thus must be more strongly bound by the B form than by N. In fact, Katz and Klotz (1953) have shown that essentially no Ca^{2+} is bound by bovine albumin at pH 5-6, whereas about 1-3 mol/mol of albumin are bound at pH 8.0 and 8.8 in the 10^{-3}-10^{-2} M free Ca^{2+} range. This is in substantial agreement with calcium binding data of Saroff and Lewis (1963) for human albumin, which was found to bind about 5 or 6 mol of calcium/mol of albumin in the pH 9-10 range (at 10^{-2} M Ca^{2+}). This immediately explains the absence of any effect of Ca^{2+} on either the optical rotatory properties or on the ^{19}F chemical shift when the protein exists in the N state. Furthermore, the model proposed by Saroff and Lewis (1963) to explain this surprising pH dependence of calcium binding is of great interest in the context of the present discussion. As they point out, the problem is not to explain strong binding at alkaline pH, but rather to account for the lack of binding at neutral pH. They proposed that calcium binding involves chelation with carboxylate, imidazolium, and ammonium groups which are engaged in ion-pair interactions in the neutral state of the protein. The decrease in chemical shift occurring at pH values above 9 (Figure 5), not seen in the absence of calcium (Figure 4), doubtless reflects the enhanced calcium binding seen by Saroff and Lewis (1963) in this pH range. Both of these calcium effects may represent a specific calcium perturbation due to binding in the vicinity of the free sulfhydryl since there seems to be no general conformational effect associated with calcium binding between pH 8 and 10 (Figure 1).

It will be surprising if the neutral transition and the associated alterations in the properties of the albumin molecule do not have some physiological significance. Clearly, this transition would enhance the hydrogen-ion buffering ability of albumin at physiological pH and would provide for calcium ion buffering as well. We also are intrigued by possible implications of the enhanced freedom of mobility of the amino-terminal portion of the polypeptide chain carrying the highly reactive sulfhydryl group. If this occurs as postulated, the role of the mercaptide group in catalyzing the postulated disulfide bond isomerization becomes readily understandable. Nikkel and Foster (1971) concluded this reaction to involve interchange between two or more disulfide bonds, the sulfhydryl group serving a purely catalytic role. Stroupe and Foster (1973) showed that the reaction is strictly intramolecular, the sulfhydryl being unable to catalyze reaction in any other molecule than that of which it is a part. They also concluded the reaction to take place *via* the B conformer. Clearly for this to occur, the catalytic sulfhydryl group must be able to move into close proximity with the disulfide participants. One wonders if this attribute of the albumin molecule might not also be of significance in the versatile binding properties of the protein, or perhaps in some special reactivity of the free sulfhydryl group.

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References

Aoki, K., and Foster, J. F. (1957), *J. Amer. Chem. Soc. 79*, 3385.

Chen, R. F. (1967), *J. Biol. Chem. 242*, 173.

Ellman, G. L. (1959), *Arch. Biochem. Biophys. 82*, 70.

Foster, J. F., Sogami, M., Peterson, A. H., and Leonard, W. J., Jr. (1965), *J. Biol. Chem. 40*, 2495.

Goldsack, D. E., and Waern, P. M. (1971), *Can. J. Biochem. 49*, 1267.

Hagenmaier, R., and Foster, J. F. (1971), *Biochemistry 10,* 637.
Harmsen, B. J. M., De Bruin, S. H., Rodrigues De Mrianda, J. F., Janssen, L. H. M., and Van Os, G. A. J. (1971), *Biochemistry 10,* 3217.
Huestis, W. H., and Raftery, M. A. (1972), *Biochemistry 11,* 1648.
Janatova, J., Fuller, J. K., and Hunter, M. J. (1968), *J. Biol. Chem. 243,* 3612.
Kaplan, L. J., and Foster, J. F. (1971), *Biochemistry 10,* 630.
Katz, S., and Klotz, I. M. (1953), *Arch. Biochem. Biophys. 44,* 351.
King, T. P., and Spencer, M. (1970), *J. Biol. Chem. 245,* 6134.
King, T. P., and Spencer, E. M. (1973), *Arch. Biochem. Biophys. 153,* 627.
Leonard, W. J. Jr., Vijai, K. K., and Foster, J. F. (1963), *J. Biol. Chem. 238,* 1984.
Lumry, R., Smith, E. L., and Glantz, R. R., (1951), *J. Amer. Chem. Soc. 73,* 4330.
Moffitt, W., and Yang, J. T. (1956), *Proc. Nat. Acad. Sci. U. S. 42,* 596.
Muirhead, H., and Greer, J. (1970), *Nature (London) 228,* 516.
Nikkel, H. J., and Foster, J. F. (1971), *Biochemistry 10,* 4479.
Perutz, M., (1970), *Nature (London) 228,* 726.
Perutz, M. F., Muirhead, H., Cox, J. M., and Goaman, L. C. G. (1968), *Nature (London) 219,* 131.
Saroff, H. A., and Lewis, M. S. (1963), *J. Phys. Chem. 67,* 1211.
Sogami, M., and Foster, J. F. (1968), *Biochemistry 7,* 2172.
Sogami, M., Leonard, W. J., Jr., and Foster, J. F. (1963), *Arch. Biochem. Biophys. 100,* 260.
Stroupe, S. D., and Foster, J. F. (1973), *Biochemistry 12,* 3824.
Vijai, K. K., and Foster, J. F. (1967), *Biochemistry 6,* 1152.
Wallevik, K. (1973), *J. Biol. Chem. 248,* 2650.
Yang, J. T., and Foster, J. F. (1955), *J. Amer. Chem. Soc. 77,* 2374.

Occurrence of D-Alanyl-(D)-*meso*-diaminopimelic Acid and *meso*-Diaminopimelyl-*meso*-diaminopimelic Acid Interpeptide Linkages in the Peptidoglycan of *Mycobacteria*†

Juana Wietzerbin,*,‡ Bhupesh C. Das,§ Jean-François Petit,‡ Edgar Lederer,‡,§ Mélina Leyh-Bouille,# and Jean-Marie Ghuysen#

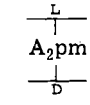
ABSTRACT: Cross-linking between peptide units in the wall peptidoglycan of *Mycobacterium smegmatis* 21732 ATCC is mediated through D-alanyl-(D)-*meso*-diaminopimelic acid and *meso*-diaminopimelyl-*meso*-diaminopimelic acid linkages occurring in a ratio of about 2:1. The occurrence of D-alanyl-(D)-*meso*-diaminopimelic acid linkages was established by the action of the *Streptomyces albus* G DD-carboxypeptidase. The occurrence of *meso*-diaminopimelyl-*meso*-diaminopimelic acid linkages was established by isolation from partial acid hydrolysates and characterization by mass spectrometric analyses of the dipeptide *meso*-diaminopimelyl-*meso*-diaminopimelic acid and the tripeptide *meso*-diaminopimelyl-*meso*-diaminopimelyl-*meso*-diaminopimelic acid. *meso*-Diaminopimelyl-*meso*-diaminopimelic acid interpeptide linkages also occur in the wall peptidoglycan of *M. tuberculosis* BCG Pasteur strain.

The wall peptidoglycan presents a remarkable consistency of structure throughout the bacterial world (Ghuysen, 1968; Schleifer and Kandler, 1972). Basically, the glycan moiety consists of linear strands of alternating β-1,4-linked pyranoside *N*-acetylglucosamine and *N*-acetylmuramic acid residues. The carboxyl groups of the muramic acid residues are linked to peptide units having the general sequence L-Ala-D-Glu(L-R_3-D-Ala).[1] The peptide units of adjacent glycan strands are, in turn, cross-linked through bridges which extend from the C-terminal D-Ala of one tetrapeptide either to the ω-amino group of the L-R_3 residue or (through a Lys or Orn molecule) to the α-carboxyl group of D-glutamic acid of another peptide unit. The location of these bridges and their composition were used to divide bacterial species into four main chemotypes (Ghuysen 1968). From previous studies, the wall peptidoglycans of Mycobacteria appeared to be built up according to the same

† From the Institut de Biochimie, Université de Paris-Sud, 91405 Orsay, France, the Institut de Chimie des Substances Naturelles, Centre National de la Recherche Scientifique, 91190 Gif sur Yvette, France, and the Service de Microbiologie, Faculté de Médecine, Institut de Botanique, Université de Liège, Sart Tilman, 4000 Liège, Belgique. *Received March 12, 1974.* The work in Orsay and Gif was supported, in part, by grants from the Fondation pour la Recherche Médicale Française and the New York Cancer Research Institute. The work in Liège was supported, in part, by the Fonds de la Recherche Fondamentale Collective, Brussels, Belgium (Contract No. 1000), and of the Institut pour l'Encouragement de la Recherche Scientifique dans l'Industrie et l'Agriculture, Brussels, Belgium (Contract No. 1699).

‡ Université de Paris-Sud.
§ Centre National de la Recherche Scientifique.
# Université de Liège

[1] Abbreviations used are: A_2pm, *meso*-diaminopimelic acid, the stereoisomery of which is represented as



R_3, third amino acid residue of a peptidoglycan (A_2pm, Lys, or Orn, etc.); N_2ph, dinitrophenyl.