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# Equilibrium Binding of Spin-Labeled Fatty Acids to Bovine Serum Albumin: Suitability as Surrogate Ligands for Natural Fatty Acids†

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ABSTRACT: Electron paramagnetic resonance (EPR) and saturation transfer EPR (ST-EPR) spectroscopies were used to characterize the binding of spin-labeled fatty acid (SLFA) to bovine serum albumin (BSA). Association constants of three stearic acid derivatives labeled with a nitroxyl radical at C-5, C-12, or C-16 were estimated by EPR spectroscopy as the ratio of SLFA to BSA was increased from about 0 to 9. The values were compared to those for unmodified stearate. With all three SLFA, it was apparent that the nitroxyl residue modified the binding pattern. For SLFA:BSA ratios up to 1, which probably involves the site(s) on BSA most specific for long-chain FA, the C-16 derivative bound with an affinity similar to that of the natural FA. At higher ratios, the association constants for this SLFA were lower than those for stearate. The C-12 and C-5 derivatives showed only low-affinity binding relative to stearate. The spectral parameter, $W$, was constant for SLFA:BSA ratios between 0 and 1 in the case of the C-16 compound, indicating physical homogeneity of the high-affinity binding site. At higher ratios, the spectra changed progressively, indicating inhomogeneity of the lower affinity binding sites although parallel changes in association constants were not observed. Changes in $W$ due to Heisenberg spin exchange were ruled out. By examining the mobility profile of the bound SLFA by both EPR and ST-EPR techniques, it was shown that the nitroxyl group was maximally immobilized when attached near the center of the carbon chain of the bound SLFA.

Spin-labeled fatty acids (SLFA)[1] have been used by Morrisett et al. (1975) to characterize fatty acid binding to serum albumin by electron paramagnetic resonance (EPR) spectroscopy. This technique permits differentiation of free and bound SLFA, as illustrated in the study by Rehfield et al. (1978), and thus appears to simplify estimation of association constants for SLFA–serum albumin complexes. Rehfield et al. (1978) also used EPR to demonstrate a difference in binding of SLFA to human and bovine serum albumins. Furthermore, since the paramagnetic nitroxide group can be located at various positions along the backbone of the fatty acid molecule, determination of the site of maximal immobilization of the BSA-bound SLFA appeared possible. In such a study, Morrisett et al. (1975) concluded that the carboxyl terminus of stearic acid was more rigidly fixed than either the

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[1] Abbreviations: EPR, electron paramagnetic resonance; ST-EPR, saturation transfer electron paramagnetic resonance; SLFA, spin-labeled fatty acids; BSA, bovine serum albumin; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; I(1,14), 16-nitroxyl stearate; I(5,10), 12-nitroxyl stearate; I(12,3), 5-nitroxyl stearate (5-NS).

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central portion of the hydrocarbon chain or the methyl terminus.

In applications of spin-labeling to biological systems, it is important to establish that the spin-label derivative is a suitable analogue of the unmodified ligand. Morrisett et al. (1975) observed that spin-labeled stearate quenched the intrinsic fluorescence of BSA in the same concentration range as observed for oleic acid, and Rehfield et al. (1978) concluded that binding of spin-labeled stearate I(12,3) to both BSA and human serum albumin was similar to that of [$^{14}$C]stearate.

In an effort to corroborate and expand these earlier studies, we have examined the binding of the three spin-labeled adducts of stearic acid to BSA, and we have made a comparison of the binding of these SLFA to that of natural long-chain fatty acids. Our results will be discussed in terms of the suitability of the SLFA as surrogates for natural fatty acids, the determination of association constants for primary and secondary binding sites, the mobility of the SLFA when bound to BSA, and the physical characteristics of the binding sites.

## Materials and Methods

*Bovine Serum Albumin and Spin-Labeled Fatty Acids.* Bovine serum albumin (BSA) was purchased from Sigma Chemical Co. (St. Louis, MO) and was specified to contain no more than 0.005% fatty acid by weight (less than 2 fatty acid molecules per 100 BSA molecules). Stock solutions (10% BSA by weight) were prepared in 0.01 M Hepes, 0.128 M NaCl, 0.0014 M $MgSO_4$, and 0.0052 M KCl. The pH was adjusted to 7.4 with NaOH. Several lots of BSA were tested and found to be identical with respect to SLFA binding. Stock solutions could be stored at −20 °C for at least 3 months. The BSA preparation was examined by column chromatography by applying 10 mL of the 10% BSA stock solution to a column (2.5 × 100 cm) of Sephadex G-150 equilibrated in the Hepes buffer. The column was developed at approximately 10 mL/h, and 3-mL fractions were collected. Protein content was estimated by its absorbance ($E^{1\%,1cm}_{280nm} = 6.10$). The elution profile was similar to that obtained by Morrisett et al. (1975). We are in agreement with these investigators, who found no spectral differences between pure monomeric BSA and the commercial preparation containing monomers and dimers.

Spin-labeled stearic acid derivatives were purchased from Syva Research Chemicals (Palo Alto, CA). The compounds used were 2-(3-carboxypropyl)-4,4-dimethyl-2-tridecyl-3-oxazolidinyloxy, 2-(10-carboxydecyl)-2-hexyl-4,4-dimethyl-3-oxazolidinyloxy, and 2-(14-carboxytetradecyl)-2-ethyl-4,4-dimethyl-3-oxazolidinyloxy. These are designated as I(12,3), I(5,10), and I(1,14), respectively. Stock solutions (0.01 M in either methanol or ethanol) were stored at 4 °C in screw-capped tubes which were wrapped in aluminum foil to minimize exposure to light. For some experiments, paramagnetic and diamagnetic nitroxides were used simultaneously. The paramagnetic nitroxide was first reduced to the diamagnetic hydroxylamine by mixing with equimolar amounts of ascorbic acid in methanol. The reduction was complete in 90 min at room temperature. Residual ascorbate was then oxidized by ascorbic acid oxidase (Boehringer) just before the addition of the SLFA to the buffer. Only the I(1,14) SLFA was employed for these experiments since it alone remained in the reduced form in the Hepes buffer; the other two SLFA were immediately air-oxidized back to the nitroxide.

*EPR Spectrometer and Accessories.* Spectral data were acquired with a Varian E-112 spectrometer equipped with phase-sensitive detection at 100 kHz for the first harmonic and second harmonic (50-kHz applied modulation) responses. The standard $TE_{102}$ microwave cavity (Varian E-231) was used for most experiments, and a $TM_{110}$ (Varian E-238) was occasionally employed. Samples were examined in the aqueous flat cell appropriate for the cavity design (S-812 for TE and S-813 for TM; James F. scanlon Co., Solvang, CA). Temperature was maintained by passage of dry nitrogen gas through a Varian E-257 temperature controller and then through the radiation slots of the cavity. Sample temperature was continuously monitored by a thermistor placed in contact with the solution itself. The temperature was 23 °C unless otherwise noted.

*Formation of SLFA–BSA Complexes.* The procedure for preparing SLFA–BSA complexes was as follows: (1) An aliquot of the Hepes buffer was placed in a test tube and stirred briskly by means of a magnetic flea. (2) The stock solution of SLFA in absolute alcohol was slowly added from a syringe (Hamilton Precision), the tip of which was placed sufficiently below the vortex to ensure that the lower density alcohol did not accumulate at the surface. Brisk stirring was continued only during addition of SLFA since prolonged stirring produced a SLFA precipitate in the center of the vortex. (3) At a reduced stirring speed, BSA from a 10% stock solution was added quickly. Stirring was stopped, and final mixing was achieved by repeated aspiration into a Pasteur pipet. Samples prepared by this method reached equilibrium in less than 2 min, and the EPR spectrum remained constant for up to 8 h at room temperature. Dilution of a SLFA–BSA solution (ratio 4:1) with unlabeled BSA to yield a final SLFA:BSA ratio of 2 was spectroscopically identical with a 2:1 SLFA:BSA solution prepared as described above. These observations suggest that complexes prepared in this fashion are at thermodynamic equilibrium. It should be noted that this method can be used with fatty acid concentrations 2–3 times above the solubility limit. It is well suited for the more soluble fatty acids such as SLFA or unsaturated fatty acids but is not recommended for stearate or palmitate. It was further noted that the quantity of alcohol necessary to make up SLFA–BSA complexes at ratios greater than 4:1 increased the fraction of fatty acid in the unbound form. This increase did not exceed 20% and did not significantly modify the interpretation of the data.

Other procedures for preparing SLFA–BSA complexes were less satisfactory. For example, addition of SLFA in alcohol to BSA solutions induced changes in line shape and progressive increases in free SLFA (up to 10-fold) over a period of 4 h, due, perhaps, to protein denaturation. Another procedure in which an aliquot of the SLFA in alcohol was added to a test tube and the solvent removed under dry nitrogen gas followed by albumin addition in Hepes buffer was also unsatisfactory. We found, as have others (Spector & Hock, 1969), that formation of complexes with a ratio greater than 2 was not possible by this technique, probably because of adsorption of the SLFA on the test tube walls.

*EPR Data Collection and Analysis.* Ordinary EPR (in phase, first harmonic absorption) spectra were recorded as either 40- or 100-G displays by employing 10-mW microwave power, 1.0-G modulation amplitude (peak to peak), and 100-kHz Zeeman modulation/detection. All ST-EPR spectra, (out of phase, second harmonic absorption) were collected as 100-G displays by using the $TE_{102}$ cavity. The flat cell was positioned parallel to the static (Zeeman) field, and the instrument was operated at 63-mW microwave power, 5.0-G modulation amplitude, and 50-kHz modulation/100-kHz detection.

The equilibrium distribution of free and bound SLFA was determined by measuring the height of the $M_I = -1$ (high-field) line which is generated almost exclusively by free SLFA

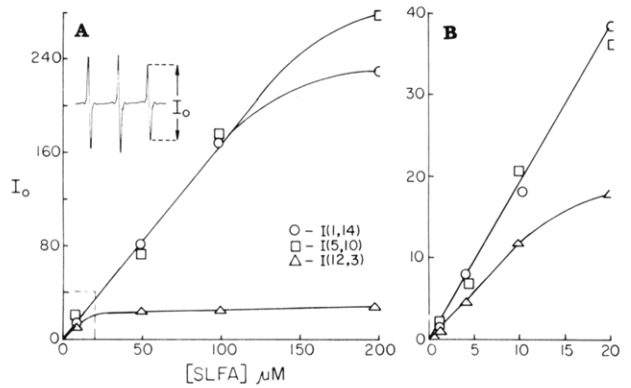


FIGURE 1: (A) Amplitude ($I_0$) of the high-field EPR peak vs. the concentration of SLFA added (in Hepes buffer at 23 °C). $I_0$ was measured in centimeters and normalized with respect to receiver gain $\times 10^{-4}$. Calibration of $I_0$ vs. free SLFA concentration was limited to the linear, initial slope of the curves, and binding experiments were restricted to those SLFA:BSA ratios which gave rise to concentrations of free SLFA in that concentration range. The flattening of the curves in the figures indicates the solubility limits of the SLFA. No micelle formation was observed. (B) Enlargement of the area enclosed by dashed lines in (A) which shows the linear portion of the solubility curve for I(12,3).

Table I: Parameters of SLFA Binding to Bovine Serum Albumin

| SLFA | no. of sites | $K_A$ ($M^{-1}$) |
|---|---|---|
| I(1,14) | 1 | $1.0 \times 10^8$ |
| | 8 | $1.5 \times 10^5$ |
| I(5,10) | 1 | $2.5 \times 10^6$ |
| | 7 | $1.8 \times 10^5$ |
| I(12,3) | 8 | $6.6 \times 10^5$ |

(Morrisett et al., 1975). The $M_I$ line was compared to standard curves of peak height vs. known free SLFA concentration (Figure 1). Binding data were collected for SLFA:BSA ratios from 0.25 to 8 which gave rise to free SLFA well below the limits of its solubility (Figure 1). The concentration of BSA by weight was 0.25%; the molecular weight was taken as 68 000 (Spector, 1975). The spectra of bound SLFA were analyzed by measuring the distance, $H$, between the outermost extrema from 100-G scans and by measuring $W$, the half-width at half-maximum height of the low-field extremum peak, from 40-G scans (Figure 2). Since the right shoulder of the low-field extremum contained a spectral contribution from free label at the higher ratios, $W$ was measured on the left of the peak. ST-EPR spectra of 0.5:1 SLFA:BSA complexes were analyzed by measuring the $C'/C$ parameter as described elsewhere (Thomas et al., 1976; Beth et al., 1979).

## Results

*Binding of SLFA to BSA.* Determinations of free and bound SLFA at different FA to BSA ratios were made for each of the SLFA and are presented as Scatchard plots (Figure 3). These data were graphically analyzed according to Scatchard (1949). It should be noted that the ordinate axis is expanded 12-fold for I(5,10) and 7-fold for I(12,3) relative to the ordinate for I(1,14). The estimates of the association constants are presented in Table I. The I(1,14) isomer appeared to bind to one high-affinity (primary) site and to a class of lower affinity (secondary) sites, whereas I(5,10) and I(12,3) appeared to bind only to the lower affinity (secondary) sites.

Competition between unlabeled fatty acid and SLFA for binding to BSA was examined as seen in Figure 4. Fatty acid–BSA complexes were prepared by using 1 mol of SLFA/BSA and up to 10 mol of oleate/mol of BSA. The

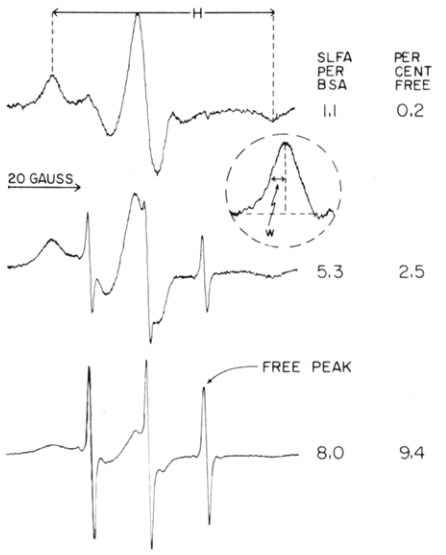


FIGURE 2: EPR spectra of I(1,14) at three ratios of FA to BSA demonstrating the increase in the high-field peak (free SLFA) relative to the low-field turnaround as the ratio is increased. For maintenance of an optimal vertical amplitude in this figure, the receiver gain was decreased as the ratio was increased. The percent of free SLFA was estimated from the height of the high-field peak which was routinely amplified to increase the precision of the measurement. The field scan sensitivity was increased (scan width decreased from 100 to 40 G) to measure the parameter $W$ (shown in inset) more accurately.

distribution of SLFA between free and bound forms was then estimated. The percentage increase in free SLFA as the oleate was raised to 4 mol/mol of protein is shown in the inset. I(1,14) was measurably displaced from BSA over the entire range of oleate concentrations, but free I(5,10) or I(12,3) could not be detected until more than 4 mol of oleate was added per mol of BSA (inset, Figure 4). As will be discussed, interpretation of these data is complex because SLFA displaced from one site by oleate can rebind to other empty sites on BSA.

*Dependence of the Spectrum of Bound SLFA on the SLFA:BSA Ratio.* The EPR spectra of bound SLFA were characterized in terms of two parameters, $H$ and $W$ (Figure 2). H increases monotonically as the motional frequency of the probe declines in the correlation time range $10^{-9}$–$10^{-7}$ (Mc Calley et al., 1972). It also increases with increasing polarity of the environment and may be affected by Heisenberg spin exchange at frequencies approaching those of the nitrogen hyperfine interactions. In this work, $H$ was found to be constant for SLFA:BSA ratios up to 8.

The parameter $W$ increases as rotational motion increases in the range of $10^{-7}$–$10^{-8}$ s and is a more sensitive indicator than $H$ for correlation times near $10^{-7}$ s. However, $W$ will also be more affected by heterogeneity of environments (i.e., nitrogen hyperfine interactions) and by Heisenberg spin exchange (i.e., by Heisenberg frequencies on the order of proton interactions) than will the parameter $H$. In this work, $W$ was observed to change markedly as the ratio of SLFA to BSA ($n$ in Figure 5) was increased from 0.25 to 8. The data were collected at several protein concentrations (0.25–2.0%) in order to make the EPR responses consistent in magnitude and precision. Changes in protein concentration at a constant SLFA:BSA ratio did not produce measurable change in $H$ or $W$.

The increase in $W$ as sites were populated was not due to Heisenberg spin exchange. Experiments performed with complexes of BSA prepared with SLFA I(1,14) and its reduced (hydroxylamine) diamagnetic analogue showed similar changes in $W$, although peak amplitudes were diminished, as

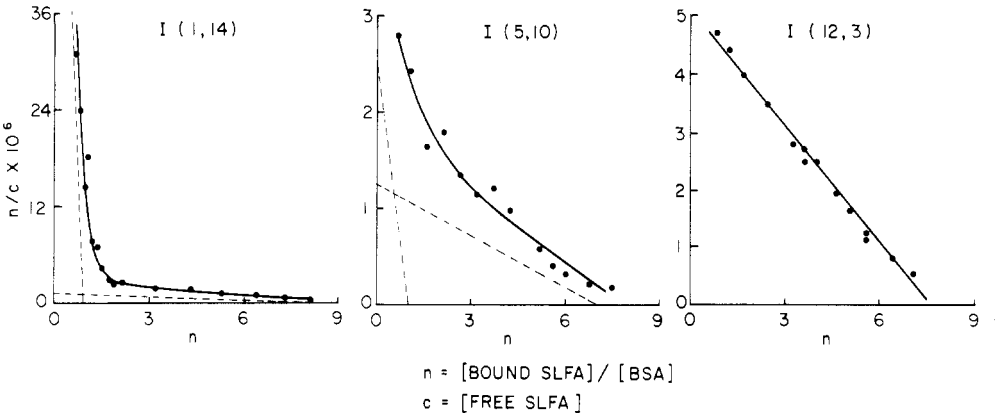


FIGURE 3: Scatchard plots of SLFA distribution between free and bound forms. Bound SLFA was estimated as the difference between total and free SLFA. The solid lines are the best (least-squares) fit to the data assuming binding to multiple classes of sites with the association constants and numbers of sites reported in Table I. The dashed lines show the individual binding to the separate classes of sites.

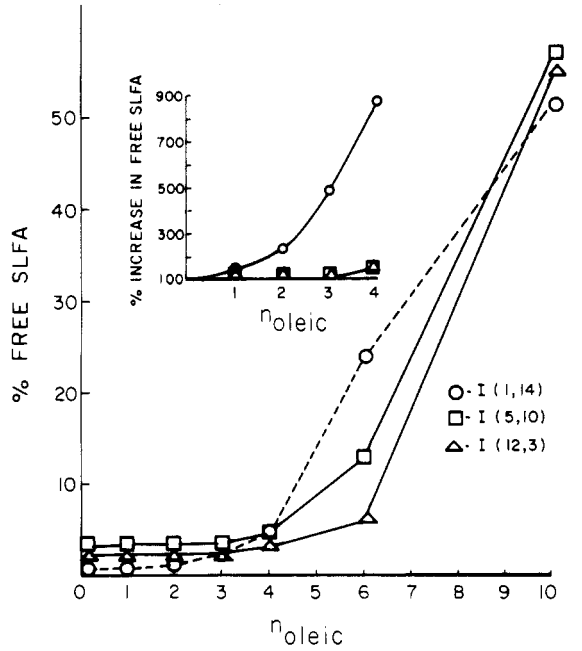


FIGURE 4: Percent of SLFA released in the presence of increasing amounts of oleic acid. The values for $n$ on the abscissa are the moles of oleate added per mole of BSA. The SLFA:BSA ratio was maintained at 1. The inset shows the percent increase in concentration of free SLFA.

expected. This would appear to eliminate Heisenberg spin exchange as a factor in the modulation of $W$. Thus, the rise in $W$ was presumably due to changes in microenvironment (e.g., polarity and steric hindrance).

A constant value for $W$ was reproducibly obtained with I(1,14) at FA:BSA ratios less than or equal to 1. Experiments in which 1:1 complexes of I(1,14)–BSA were diluted with unlabeled BSA confirmed this constancy. These data are consistent with the Scatchard plots and indicate homogeneity of the high-affinity binding site.

*Mobilities of Bound SLFA.* Morrisett et al. (1975) suggested that the segment of the FA chain close to the carboxyl terminus was bound most tightly to the BSA. They based this suggestion on the observation that $H$ was greater when the nitroxyl radical was attached to carbon 5 [as in I(12,3)] than when the label was on carbon 12 or 16. We confirmed their observation but found, however, that $W$ was largest for I(12,3), suggesting that the label was most mobile on carbon 5. In an effort to resolve this ambiguity, we measured the motionally independent, intrinsic or powder values of $H$ and $W$.

As noted earlier, any comparison of mobilities between SLFA must consider the effects of binding site polarity and Heisenberg spin exchange. Mc Calley et al. (1972) demonstrated that $\Delta H$ (where $\Delta H = H_\infty - H$) was a reliable measure of probe mobility and was relatively insensitive to changes in polarity and intramolecular Heisenberg spin exchange. In agreement with Freed (1976), we found that $\Delta W$ (where $\Delta W = W - W_\infty$) also reflects probe mobility and is reasonably independent of polarity and intramolecular Heisenberg spin exchange effects. $H_\infty$ and $W_\infty$ are intrinsic or "powder" values and are observed when molecular motion is slower than the EPR rigid lattice limit. We prepared SLFA–BSA complexes, therefore, in 30% glycerol by weight and lowered the temperature to −15 °C, at which point $H$ maximized and $W$ minimized ($H_\infty$ and $W_\infty$, respectively; Table II). The plots of $\Delta H$ and $\Delta W$ showed that the two estimates of EPR mobility

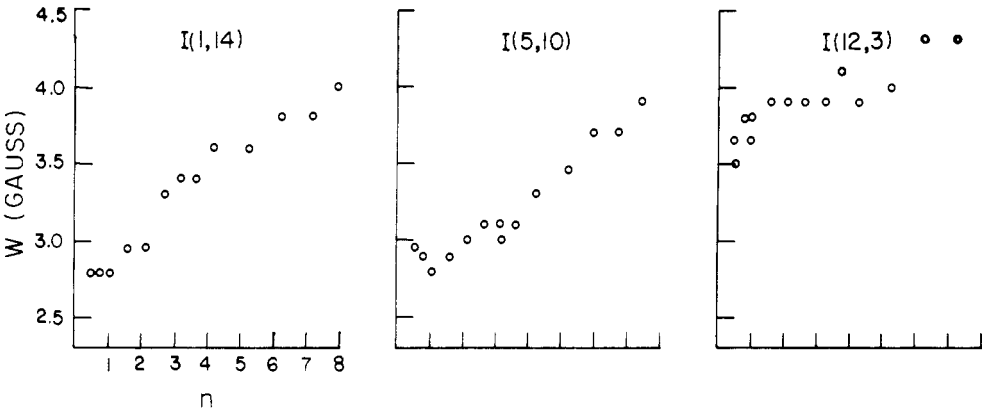


FIGURE 5: Dependence of the EPR parameter $W$ on the molar ratio of the SLFA–BSA complex ($n$).

Table II: Values of the EPR Parameters,[a] $H$ and $W$, for the SLFA[b]

| SLFA[b] | $H_\infty$ | $H$ | $\Delta H$ | $W_\infty$ | $W$ | $\Delta W$ |
|---|---|---|---|---|---|---|
| I(1,14) | 66.75 | 60.60 | 6.15 | 2.30 | 2.80 | 0.50 |
| I(5,10) | 66.75 | 62.25 | 4.50 | 2.70 | 2.9–2.95 | 0.2–0.25 |
| I(12,3) | 69.25 | 63.00 | 6.25 | 3.15 | 3.5–3.65 | 0.35–0.50 |

[a] $H$ and $W$ are values at 23 °C in Hepes buffer while $H_\infty$ and $W_\infty$ are values at −15 °C in 30% glycerol by weight. $\Delta H = H_\infty - H$ and $\Delta W = W - W_\infty$. [b] The SLFA:BSA ratio was 0.5.

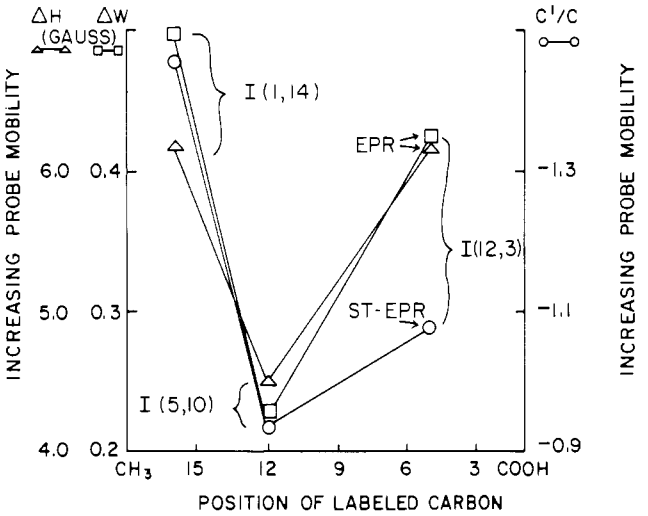


FIGURE 6: Apparent mobility of bound SLFA as a function of the position of the spin-label on the fatty acid carbon chain. EPR mobility indicators, $H$ and $W$, were obtained from Table II. The SLFA:BSA ratio was 0.5:1.

were in agreement (Figure 6). As indicated in the figure, the EPR data were confirmed by measurement of the ST-EPR parameter, $C'/C$. This parameter is known (Thomas et al., 1976; Beth et al., 1979) to decrease as mobility increases. Thus, the nitroxyl radical appears to be most immobilized when it is near the center of the FA chain. This interpretation, however, is subject to important reservations, as will be discussed.

## Discussion

EPR spectroscopy provides a technique for the study of ligand binding to proteins. It is especially advantageous for poorly water-soluble ligands such as fatty acids for which separation techniques are tedious. Free and bound ligand can be differentiated without separation so that distribution may be quickly and easily quantitated. Furthermore, the spectrum of the bound ligand may suggest characteristics of the binding site with respect to conformation, polarity, and proximity to other binding sites. However, since the spin-labeled substance is chemically modified, its binding may differ from that of the native compound.

*SLFA Suitability.* The effect of the nitroxyl radical on binding is apparent from the different binding constants obtained when the label is attached at different points along the FA chain (Table I). I(1,14) appears to be most similar to unmodified stearate in binding characteristics. There is apparently one high-affinity site on BSA for I(1,14) (Figure 3) with an association constant (Table I) of about $1 \times 10^8$. Spector & Fletcher (1978) report measurements of [$^{14}$C]-stearate binding, but their studies were carried out at 37 °C whereas ours were made at 23 °C. As judged by the behavior of palmitate binding at these two temperatures (Spector et al., 1969), the association constants for a long-chain FA at 37 °C would be about 4-fold higher at 23 °C to give a value of about $5 \times 10^8$ for stearate. This value is reasonably close to the association constant for I(1,14). On the other hand, the association constants for the "secondary" binding sites, 2–9, for SLFA are about 2 orders of magnitude lower for I(1,14) than for [$^{14}$C]stearate and are uniform in magnitude whereas they decrease progressively for the unmodified ligand (Spector & Fletcher, 1978). Thus, the nitroxyl residue would appear to interfere significantly with binding to the secondary sites. The two highest affinity sites have been suggested to be specific for FA (Spector & Fletcher, 1978) whereas the lower affinity sites bind additionally a wide variety of organic compounds. A more precise and detailed comparison between binding of the spin-labeled and natural stearate would not appear possible, since neither EPR spectroscopy nor the equilibrium method with $^{14}$C-labeled FA (Spector & Fletcher, 1978) can give precise values for binding to individual sites. A major difficulty in this regard is that each increment of ligand distributes over multiple sites on BSA rather than filling each new site in a stepwise fashion (Spector & Fletcher, 1978).

It would appear probable from Figure 3 that I(5,10) and I(12,3) do not occupy the highest affinity sites for FA binding, but such a conclusion is not certain. It is possible that these sites are occupied but with lower association constants because of hindrance by the nitroxyl radical. I(1,14) was the only SLFA which gave rise to measurable unbound FA when oleate in an amount up to 4 mol/mol of BSA was added to a 1:1 SLFA:BSA complex (Figure 4). This supports the view that only I(1,14) is bound to (and hence displaced from) the high-affinity site among the three SLFA. However, the affinity for rebinding of I(1,14) was slightly lower than for the other two SLFA (Table I). Thus, I(5,10) and I(12,3) may have bound to the highest affinity sites on BSA and been displaced by oleate, but the concentration of unbound SLFA did not rise because the ligands rebound to empty sites on BSA more tightly than did I(1,14). In conclusion, spin-labeling in any of the three positions on the FA chain modifies binding to BSA substantially. The data suggest that of the three commercially available SLFA, I(1,14) is the most similar to the natural ligand in its binding to the highest affinity site on BSA.

*Spectral Variations of the Bound Ligand.* The parameter $W$ (Figure 2) was observed to depend strongly on the number of SLFA bound (Figure 5). The relatively high affinity of the first 1 binding site for I(1,14), as seen in Figure 3, was also expressed in the spectrum of bound ligand by a constant value of $W$ when 0–1 mol of I(1,14) was bound per mol of BSA. The constancy would reflect the competitive advantage of this site relative to secondary sites as well as the physical homogeneity of the SLFA–protein complex in the range of ratios below 1. Using the observed association constants, we calculated that 89% of I(1,14) would be bound on the high-affinity site at a FA:BSA ratio of 1. The algorithm for this calculation is given in detail elsewhere (Abumrad et al., 1981).

As the number of SLFA molecules per molecule of BSA was increased from 1 to 8, $W$ was observed to rise. This could be caused by an increase in Heisenberg spin exchange, bound–free ligand exchange, mobility of the bound probe, or environmental polarity changes. Increased Heisenberg spin exchange results when spin-labels approach each other very closely as additional binding sites are populated. This possibility was eliminated by observing that the inclusion of the diamagnetic reduced, hydroxylamine I(1,14) analogue caused an increase in $W$ equal to that induced by an equivalent addition of the paramagnetic I(1,14) nitroxide. Furthermore,

complexes prepared from mixtures of I(1,14) and oleic acid also exhibited an increase in $W$ as the ratio of the bound ligand (SLFA plus oleic) to protein was increased. The contribution of bound–free ligand exchange to $W$ cannot be assessed from the EPR data but is probably negligible. Dissociation rate constants for oleate–human serum albumin (HSA) complexes have been measured to be approximately $3 \times 10^{-2}$ $s^{-1}$ (Scheider, 1980). A line-width contribution of this magnitude is insignificant compared to the intrinsic line width which conservatively is $10^{-7}$ $s^{-1}$ (i.e., $1/T_2 = 10^7$ $s^{-1}$ + $10^{-2}$ $s^{-1}$ $\approx 10^7$ $s^{-1}$).

Although a precise physical interpretation cannot be made, the changes in $W$ indicate an inhomogeneity of the low-affinity binding sites which is not reflected in detectable changes in the association constants.

*Mobility of Bound SLFA.* Figure 6 summarizes in graphic form the estimates of mobility of the nitroxyl radical attached to different positions along the carbon chain of stearate when bound to the highest affinity site(s) on BSA. The estimates by three different experimental approaches are in agreement and suggest that the central portion of the bound SLFA is more rigidly attached to BSA than is either terminus. This differs from the suggestion of Morrisett et al. (1975) that the SLFA is bound to the protein near the carboxyl end of the fatty acid chain. Either suggestion, however, would be valid only if the three SLFA bound to the same site(s) on the BSA. As discussed above, this is not established because of the uncertainties in interpreting differences in binding affinities. Furthermore, suggestions with regard to segmental binding of SLFA to binding of the natural FA are subject to the additional problem that the steric hindrance of the nitroxyl radical is maximal in the segment of FA for which information regarding mobility is sought.

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# Flexibility of Myosin Rod Determined from Dilute Solution Viscoelastic Measurements†

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ABSTRACT: The frequency dependences of the storage and loss shear moduli, $G'$ and $G''$, of myosin rod solutions at 1.0 and 7.0 °C were measured by use of the Birnboim-Schrag multiple lumped resonator apparatus in solvents with and without glycerol. The infinite dilution moduli were determined and compared with theoretical models for a rigid rod and a freely jointed trinodular rod and with an empirical model for a semiflexible rod. Only the latter could fit the data. A rotational relaxation time of 25 μs and a slowest bending time of 3.1 μs, both reduced to water at 20 °C, were determined from the fit. A persistence length of about 130 nm was obtained from either the bending time, the rotational relaxation time, or the intrinsic viscosity. The average thermal excursion of the end of subfragment 2 was estimated to be 26 nm, more than sufficient to span the gap between the thick and thin filaments in muscles at all sarcomere lengths. Thus, a hinge between heavy meromyosin and light meromyosin does not appear necessary for myosin–actin contact. Young's modulus of about $1 \times 10^9$ $N/m^2$ also makes it unlikely that subfragment 2 can be the elastic element in the Huxley–Simmons model of muscle contraction.

Current theories of muscle contraction suppose that force generation occurs through an interaction between myosin in the thick filaments and actin in the thin filaments (Harrington, 1979a). The detailed mechanism of force generation is not well understood but is hypothesized to involve either a rotation of the myosin heads when they are attached to the thin filaments (Huxley & Simmons, 1971) or a helix–coil transition in the helical part of myosin (Harrington, 1979b). Both models involve a step in which the myosin heads swing out from their resting position in the vicinity of the surface of the thick filaments and become attached to the thin filaments. The distance between the myosin head at rest and the surface of the thin filament is believed to be about 5–6 nm (Harrington, 1979a). Thus, a substantial radial movement would be re-

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