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Fatty acid binding sites of serum albumin probed by non-linear spin-label EPR

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Abstract

A novel form of non-linear EPR spectroscopy, viz. the first harmonic absorption spectrum recorded in phase quadrature with respect to the Zeeman field modulation, is used here to investigate spin-lattice relaxation enhancements of nitroxide spin labels bound to serum albumin that are induced by spin–spin interactions with aqueous paramagnetic ions. The advantage of this EPR method is that it is directly sensitive to spin-lattice relaxation and affected relatively little by other spectral parameters (Livshits et al., J. Magn. Reson. 133 (1998) 79–91). Relaxation enhancements by ferricyanide of bound fatty acids (*n*-SASL) spin-labelled at different positions, *n*, in the chain are compared with those of different maleimide spin label derivatives attached at the single free –SH group, as well as with those of the spin labels free in solution. It was found that: (1) the encounter frequency of ferricyanide with 5-SASL and 12-SASL bound to serum albumin is more than two times less than that with 16-SASL; (2) the accessibility of ferricyanide to 16-SASL is comparable to that of the more immobilised covalently bound spin labels; and (3) the absolute values of the encounter frequencies for the bound spin-labelled fatty acids are approximately a factor of ten smaller than for the corresponding free spin labels, but the latter show a dependence on position of labelling that is similar to the bound labels. A kinetic scheme that is consistent with these relative differences involves rapid reversible transitions between an 'open' and 'closed' state, in which interaction with aqueous paramagnetic agents is possible only in the 'open' state. The equilibrium strongly favours the 'closed' state, which is further enhanced at low temperatures.

Keywords: Serum albumin; Fatty acid; Spin label; Non-linear EPR; Paramagnetic relaxation

1. Introduction

Albumin is the most abundant protein in blood plasma and is known to bind a variety of biological substrates and drugs [1]. In particular, albumin is the major transport protein for fatty acids. Several studies have focused on the nature and location of the fatty acid binding sites on albumin [2–9]. It is found that there are five principal sites for long-chain fatty acids, with different affinities that depend on the chain length of the fatty acid. For stearates, stepwise association constants range from 1.5×10^8 to 5×10^6 M^{-1}, so that no single site dominates. Even at mole ratios less than one fatty acid/protein, the fatty acids are distributed over several sites [2,4].

^{13}C-NMR studies have indicated that the bound fatty acid molecules are capable of rapid internal motions, in which the motion of the terminal methyl

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carbon is less restricted than that of the methylene carbons near the polar head of the fatty acid molecule [4].

Recently, a crystal structure was published of human serum albumin complexed with five myristic acid molecules, at a resolution of 2.5 Å [6]. This provides a structural basis for understanding the character of fatty acid binding to albumins. The fatty acid binding sites are located in different domains of the protein and have different orientations and amino acid environments. Yet all of these sites have certain common features: in each case, the hydrocarbon tail of the fatty acid is accommodated inside a deep hydrophobic cavity (pocket), while the carboxyl moiety is liganded by two or three basic or polar residues.

These data refer to a static picture of the protein in a crystal lattice. It is of considerable interest to compare this data with dynamic information on the accessibility of the bound fatty acid molecules to solvent, for the protein in solution. To this end, the spin-lattice relaxation enhancement of spin-labelled fatty acids bound to bovine serum albumin (BSA) that is induced by Heisenberg spin exchange interaction with a water-soluble paramagnetic relaxation agent is studied here. The primary goal is to compare the accessibility for different parts of the bound fatty acid molecule. Therefore, doxyl stearic acids with the nitroxide moiety attached to the hydrocarbon chain at the 5, 12 or 16 positions with respect to the carboxyl group are used. As mentioned earlier, selective binding of fatty acids to a particular site is difficult to attain [3]. However, because the above features are common to all binding sites, selective binding is not essential.

In addition to the non-covalent fatty acid binding sites, BSA has an important single cysteine residue that can be chemically modified by different alkylating reagents. In order to probe the topography of this site we also study the Heisenberg exchange interaction of the paramagnetic relaxation agent, potassium ferricyanide, with three maleimide spin labels of different lengths covalently bound to the cysteinyl thiol group.

Newly developed non-linear EPR methods [10,11] are used here to study the accessibility of the spin-labelled ligands, covalently or non-covalently bound to bovine serum albumin, to a water-soluble paramagnetic relaxation agent. Conventional EPR spectroscopy has been used previously to probe protein topography by studying Heisenberg exchange interactions between covalently bound spin labels and paramagnetic ions or complexes in solution [12]. This earlier method is based on measuring the line broadening induced by exchange interactions, and is readily applicable to weakly immobilised spin labels that are located on the protein surface and thus have relatively narrow EPR spectral lines. However, for strongly immobilised spin labels that are located in cavities or hydrophobic pockets and give broad-line EPR spectra, the detection of spin exchange would require very high concentrations of paramagnetic broadening reagent. In this case, measuring spin-lattice relaxation enhancement, as is done here, is evidently a more preferable approach. The first harmonic absorption V'_1-EPR spectra that are recorded 90° out-of-phase with respect to the magnetic field modulation are used. This is because, of the various non-linear EPR spectroscopies, this method is the most sensitive to spin-lattice relaxation and least sensitive to other parameters such as T_2-relaxation and molecular rotational motion [10]. The application here to investigating the fatty acid binding sites of serum albumin is a significant biophysical illustration of this new non-linear EPR method.

2. Materials and methods

2.1. Materials

Maleimide spin labels: 3-maleimido-proxyl (**I**), 3-(maleimido methyl)-proxyl (**II**), 3(2-[maleimido-ethoxy]-ethyl carbamoyl)-proxyl (**III**), and bovine serum albumin (BSA) were from Sigma (St. Louis, MO). Spin-labelled stearic acids, *n*-SASL (*n*-(4,4-dimethyloxazolidine-*N*-oxyl)stearic acids, with $n = 5$, 12, 16) were synthesised according to ref. [13].

2.2. Spin labelling

Covalent labelling of BSA with the spin-labelled maleimide derivatives **I–III** was performed as follows. Maleimide spin label in ethanol solution (1% v/v) was added to the protein in 100 mM phosphate buffer pH 7.0 at a 5-fold molar excess. The mixture

was incubated for 3 h at 25°C under intensive vortexing. The spin-labelled protein was separated from free spin label on a column of Sephadex G-25 (Pharmacia, Malmö, Sweden) equilibrated and eluted with the same buffer. The final concentration of the spin-labelled BSA was approximately 0.2 mM and that of the $K_3Fe(CN)_6$, when added, was 6 mM.

Doxyl stearic acids, *n*-SASL, were dissolved in methanol and dried in a nitrogen atmosphere. A 1-mM BSA solution in pH 7.0 phosphate buffer was then added to give a 5:1 molar excess of spin label. The mixture was shaken for 3 h at 25°C. These labelling conditions ensure almost complete occupancy of the different fatty acid binding sites, without the presence of any free fatty acid spin label (as evidenced from the conventional EPR spectra). The spin-labelled protein solutions were diluted 1.4 times with buffer or with 7.5 mM $K_3Fe(CN)_6$. All samples were saturated with argon.

Measurements in the presence and absence of the relaxation agent, $Fe(CN)_6^{3-}$, were performed on samples derived from the same stock spin-labelled protein, in order to avoid any variability in relaxation properties arising from possible small differences in the level of spin-labelling.

2.3. EPR spectroscopy

EPR spectra were recorded on a Varian Century Line, or on a Bruker EMX, 9 GHz EPR spectrometer at magnetic field modulation frequencies of 100 and 25 kHz over a scan range of 100–200 G, depending on the spectral extent at high microwave power. Samples of 5 mm length were contained in sealed 0.7- or 1-mm i.d. glass capillaries which were accommodated within a standard quartz EPR tube that contained light silicone oil for thermal stability. The samples were centred vertically in the microwave cavity. The entire sample chamber was sealed under argon. Temperature of the samples was controlled by nitrogen gas flow through a quartz dewar and measured by a fine-wire thermocouple located within the capillary in contact with the sample. Critical coupling conditions were used. Modulation phase settings were performed by the self-null method or by use of a non-saturated reference sample, a crystal of copper sulfate, as described in [10]. The root-mean-square microwave field, H_1, at the sample was calibrated by using a peroxylamine disulfonate solution as reference, as is described in ref. [14].

The non-linear first-harmonic absorption spectra of the spin labels were analysed by determining the double-integrated intensity of the 90° out-of-phase V'_1-EPR spectrum relative to that of the in-phase V_1-EPR spectra [10]. Calibrations of this parameter in terms of the spin-lattice (T_1) relaxation time of the spin label have been established previously by simulation of the non-linear spectra at saturating microwave H_1-fields [11]. The out-of-phase to in-phase intensity ratio, ρ'_1, is given by:

$$\rho'_1 = \frac{\int\int V'_1 \cdot d^2H}{\int\int V_1 \cdot d^2H} = \rho'^{o}_1 + \frac{a'_1 T_1^m}{1 + b'_1 T_1^m} \quad (1)$$

where the calibration constants ρ'^{o}_1, a'_1, b'_1 and the exponent m are given in ref. [11]. Similarly, the ratio $\rho'_1(M_I)$ of the out-of-phase and in-phase amplitudes of the M_I-hyperfine manifold was also used:

$$\rho'_1(M_I) = \frac{V'_1(M_I)}{V_1(M_I)} = \rho'^{o}_1(M_I) + \frac{a'_1(M_I) T_1^m}{1 + b'_1(M_I) T_1^m} \quad (2)$$

where the corresponding calibration constants $\rho'^{o}_1(M_I)$, $a'_1(M_I)$, $b'_1(M_I)$ and the exponent m are also given in ref. [11]. The amplitude form is particularly useful for determination of relaxation enhancements from paramagnetic ions that have a background EPR signal at around room temperature, which makes the precise determination of the spin label spectral integrals difficult.

3. Results

3.1. Maleimide spin labels *I–III* covalently bound to BSA

The conventional (low-power) EPR spectra of spin labels **I** and **II** (Fig. 1A) correspond to the slow-motion region, and are indicative of strong immobilisation of spin labels attached to the protein, with a small admixture of EPR signals from weakly immobilised spin labels. The EPR spectrum of spin label **III** that has a long ethoxy-ethyl carbamoyl linker corresponds to the rapid motion region (see Fig.

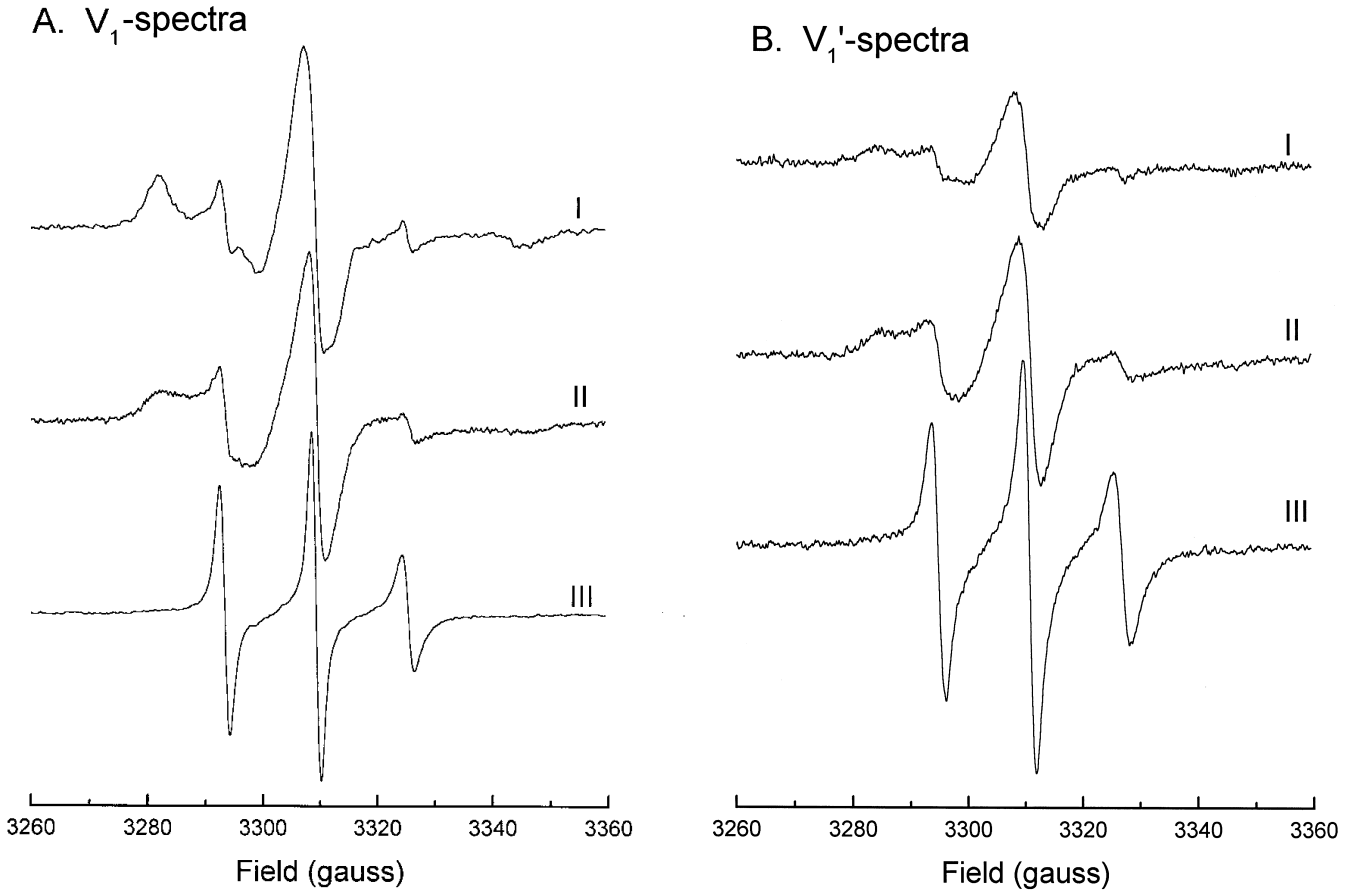


Fig. 1. EPR spectra of maleimide spin labels **I–III** covalently bound to BSA. (A) Conventional, low-power V_1-EPR spectra. (B) First-harmonic, out-of-phase V'_1-EPR spectra recorded with $H_1 = 0.5$ G and a modulation frequency of 100 kHz. Temperature 20°C, phosphate buffer, pH 7.0.

1A). Previously [15], the rotational correlation time (τ_R), and limiting values ($2A'^{\infty}_{zz}$) of the outer hyperfine splitting corresponding to the absence of protein rotation, were determined for spin label **I**. (Extrapolation to infinite viscosity was used to determine $2A'^{\infty}_{zz}$). The separation of the outer extrema, $2A'^{\infty}_{zz}$, for spin label **II** is equal, to within the error limits, to that for spin label **I**. Therefore the same values of τ_R and $2A'^{\infty}_{zz}$, were assumed for spin label **II** as for spin label **I**. The isotropic value of τ_R for spin label **III** was estimated from the differential broadening of the three hyperfine lines to be 0.55 ns [16].

The non-linear out-of-phase first-harmonic V'_1-EPR spectra of spin labels **I–III** covalently bound to BSA are given in Fig. 1B. Because the EPR spectra of **I** and **II** contain admixtures from the weakly immobilised spin labels, the ratios of the amplitudes of the low-field, $\rho'_1(M_I = +1)$, and central, $\rho'_1(M_I = 0)$, components of the out-of-phase and in-phase spectra, rather than those of the double integrals, were used for the T_1 determinations (see refs. [10,11]). These parameters were determined at the same saturating value of H_1 (0.5 G), with a magnetic field modulation frequency of 100 kHz, for spin-labelled BSA solutions in buffer and in the presence of 6 mM $K_3Fe(CN)_6$. The results are given in Table 1.

Spin-lattice relaxation times and relaxation enhancements, $\Delta(1/T_1)$, were determined from the first-harmonic out-of-phase/in-phase ratios, $\rho'_1(M_I)$, for each spin label by using the calibrations given in ref. [11]. For use in the calibrations, the rotational correlation times τ_R were determined as above, and the homogeneous (Lorentzian) line widths ($1/\gamma_e T_2$) were obtained from Gaussian–Lorentzian deconvolution of the low-field spectral components of the low-power EPR spectra. The values of $1/\gamma_e T_2$ are approx-

imately 2.4 G for **I** and **II**, and 0.5 G for **III**. The resulting relaxation enhancements, $\Delta(1/T_1)$, induced by 6 mM $K_3Fe(CN)_6$ are given for spin labels **I–III** in Table 1. Although the values of $\Delta(1/T_1)$ derived from the two spectral parameters [$\rho'_1(M_I=+1)$ and $\rho'_1(M_I=0)$] differ somewhat, the relaxation enhancement by spin exchange interaction with potassium ferricyanide is consistently the least for spin label **I**, is slightly greater for spin label **II**, and is more than two times greater for spin label **III**. The last column of Table 1 gives the averaged second order spin exchange rate constants, k_{ex}, that are calculated from the values of $\Delta(1/T_1)$ for each spin label. The spin-exchange rate constant, k_{ex}, is related to the paramagnetic relaxation enhancement by:

$$\Delta(1/T_{1,2}) = k_{ex}c_m \quad (3)$$

where c_m is the concentration of paramagnetic relaxant and $\Delta(1/T_{1,2})$ is the enhancement in T_1- (or T_2-) relaxation rate. For comparison, the values of k_{ex} that were determined for labels **I–III** free in solution from the line broadening of the low-power EPR spectra are equal within error limits: $k_{ex}(\text{free}) = 0.75 \pm 0.05 \times 10^9$ M^{-1} s^{-1}. The relative values of $\Delta(1/T_1)$ or $\langle k_{ex} \rangle$ are seen to correlate qualitatively with the length of the linker that connects the spin label proxyl group with the thiol attachment group. Alternatively, one can interpret the differences in values of k_{ex} in terms of the solid angle around the spin label that is accessible for $K_3Fe(CN)_6$. This angle is equal to 2π for the free labels, and is effectively in the region of 0.24π, 0.31π, and 0.64π, for **I**, **II** and **III**, respectively, when covalently attached to BSA. Note

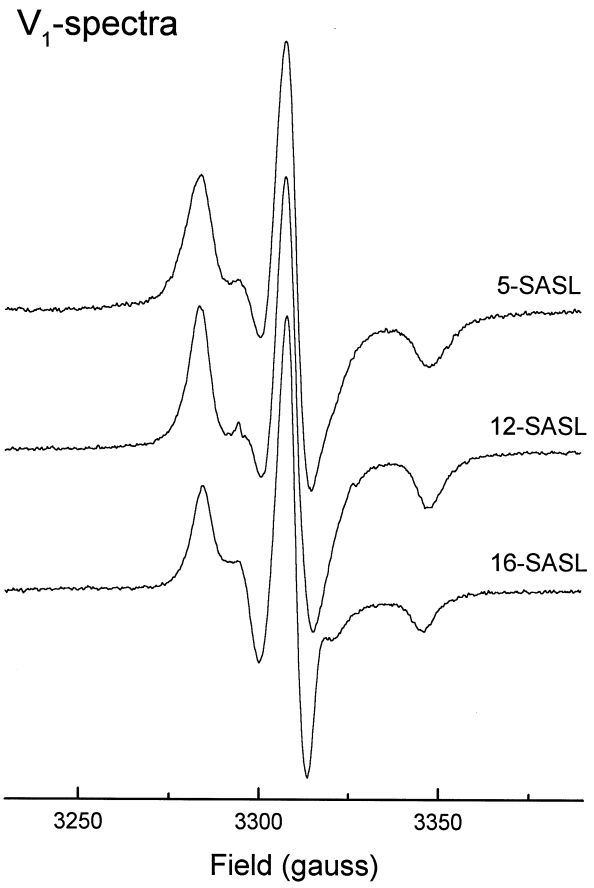


Fig. 2. Conventional low-power V_1-EPR spectra of stearic acid spin labels 5-SASL, 12-SASL, and 16-SASL non-covalently bound to BSA. Temperature 20°C, phosphate buffer, pH 7.0.

Table 1
Spin-lattice relaxation rate enhancements, $\Delta(1/T_1)$, by ferricyanide, and second-order spin-exchange rate constants, k_{ex}, for maleimide spin labels **I–III** covalently bound to BSA, as deduced from the spectral parameters, $\rho'_1(M_I)$, of the out-of-phase and in-phase first-harmonic EPR spectra at a modulation frequency of 100 kHz[a]

Spin label	Medium	$\rho'_1(M_I=+1)$	$\Delta(1/T_1)\times10^{-6}$ (s^{-1})	$\rho'_1(M_I=0)$	$\Delta(1/T_1)\times10^{-6}$ (s^{-1})	$k_{ex}\times10^{-9}$ (M^{-1} s^{-1})
I	Buffer	0.15		0.30		
	+6 mM $K_3Fe(CN)_6$	0.094	0.49	0.167	0.59	0.09
II	Buffer	0.133		0.25		
	+6 mM $K_3Fe(CN)_6$	0.067	0.59	0.12	0.79	0.11
III	Buffer	0.238		0.347		
	+6 mM $K_3Fe(CN)_6$	0.08	1.53	0.11	1.4	0.24

[a] T_1 calibrations are from ref. [11].

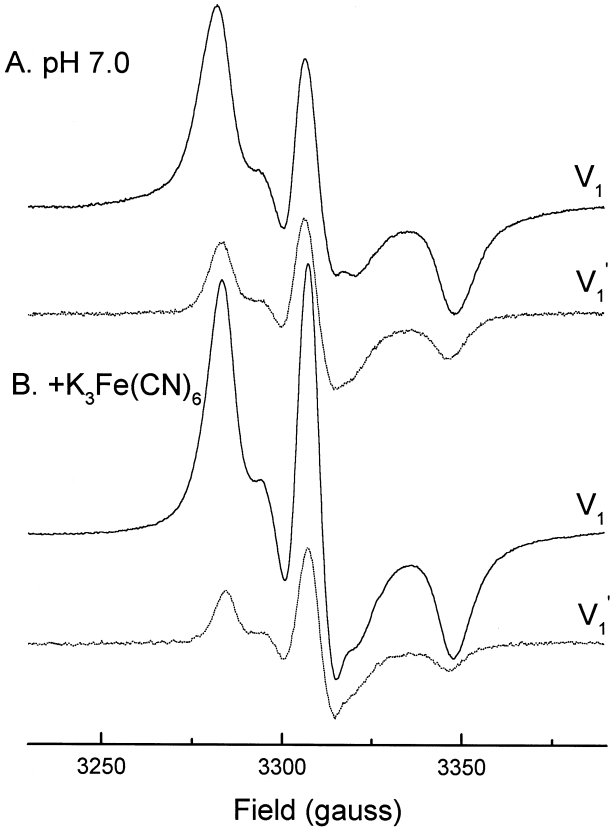


Fig. 3. Conventional V_1-EPR spectra and first-harmonic out-of-phase V'_1-EPR spectra of 5-SASL bound to BSA at 22°C, in the absence (A) and in the presence (B) of 7.5 mM $K_3Fe(CN)_6$, in phosphate buffer, pH 7.0. Modulation frequency 25 kHz and $H_1 = 0.5$ G.

that the linear dependence on concentration, c_m, of relaxant that is given in Eq. 3, tacitly includes all spin–spin interactions (e.g. dipolar, in addition to exchange) between relaxant and spin label. Collectively, it is a measure of mutual accessibility, although collisional-induced exchange is likely to dominate.

3.2. Spin-labelled stearic acids non-covalently bound to BSA

The conventional (low-power) EPR spectra of spin labels, 5-SASL, 12-SASL and 16-SASL, bound to BSA (see Fig. 2) also correspond to the slow-motion region, hence, to a strong immobilisation of the spin labels by the protein. The outer extrema separations, $2A'_{zz}$, for 5-SASL and 12-SASL are approximately the same as that for spin label **I**: 63.5 ± 0.15 G, in agreement with data published previously [8,9]. The effective isotropic rotational correlation time (τ_R) for the bound spin labels estimated from this and the limiting values of $2A_{zz}$ [17] is approximately 30 ns. This value is in agreement with the average value of τ_R predicted from hydrodynamic models for an ellipsoidal BSA molecule and with the values of τ_R determined earlier both for spin-labelled fatty acids [9] and a covalently bound spin label [18]. This means that the hyperfine tensor component, $2A_{zz}$, of the EPR spectra is not averaged by axial motion of the bound spin label that is manifested in the ^{13}C NMR spectra [4].

The value of the outer hyperfine splitting, $2A'_{zz}$, is appreciably lower for bound 16-SASL (61.5 ± 0.15 G) than for the other two spin-labelled stearic acids, 5-SASL and 12-SASL. This difference is most prob-

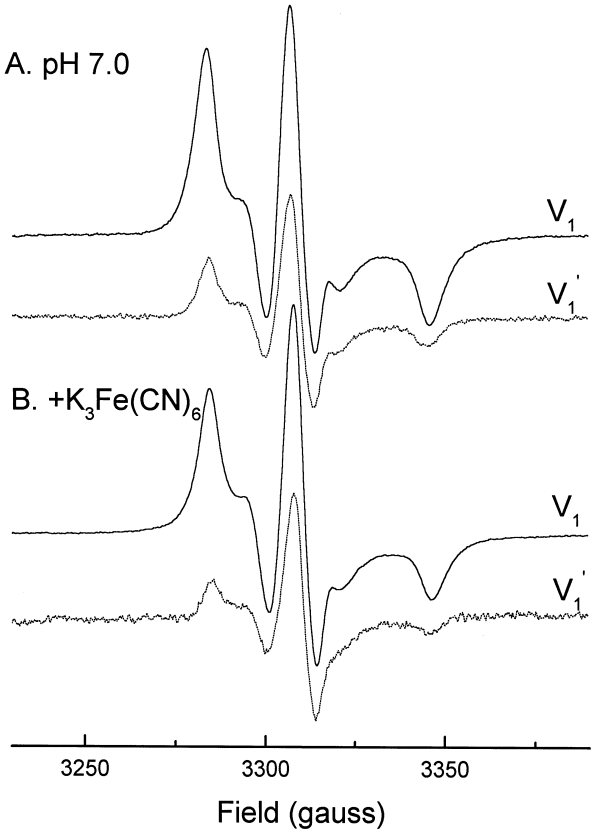


Fig. 4. Conventional V_1-EPR spectra and first-harmonic out-of-phase V'_1-EPR spectra of 16-SASL bound to BSA at 22°C, in the absence (A) and in the presence (B) of 7.5 mM $K_3Fe(CN)_6$, in phosphate buffer, pH 7.0. Modulation frequency 25 kHz and $H_1 = 0.5$ G.

Table 2
Intrinsic spin-lattice relaxation times (T_1) for 12-SASL bound to BSA determined from the low-field amplitude ratio, $\rho'_1(M_I = +1)$, of the out-of-phase and in-phase first-harmonic EPR signals at a modulation frequency of 25 kHz and different microwave field amplitudes, H_1 [a]

H_1 (G)	$\rho'_1(M_I = +1)$	T_1 (μs)
0.53	0.192	3.3
0.42	0.162	3.3
0.334	0.128	3.2
0.266	0.093	3.2
0.211	0.068	3.3

[a]Temperature 22°C, pH 7.0. Calibrations are taken from ref. [11].

ably due to the partial averaging of the $2A_{zz}$ component by residual motion of the terminal part of the fatty acid molecule that is evident from ^{13}C-NMR [4]. A similar conclusion was also reached from simulations of the conventional EPR spectra from 16-SASL bound to BSA [8].

Relaxation enhancements by ferricyanide were determined from the integrated intensity ratios, ρ'_1, as well as the amplitude ratios, $\rho'_1(M_I)$, of the out-of-phase to in-phase first-harmonic EPR spectra. The H_1-dependence of these parameters was investigated first. Such a dependence of the T_1-values for 12-SASL that is obtained from the low-field spectral amplitude ratio, $\rho'_1(+1)$, is presented in Table 2. This shows that measurements at different microwave field intensities yield essentially the same T_1 values, hence demonstrating the consistency of the T_1-calibrations given in ref. [11].

Typical conventional V_1-EPR spectra and non-linear V'_1-EPR spectra of the 5-SASL stearic acid spin label bound to BSA in the presence and absence of ferricyanide that were recorded at a saturating microwave field of $H_1 = 0.5$ G are given in Fig. 3. For comparison, corresponding conventional and non-linear EPR spectra of the 16-SASL stearic acid spin label bound to BSA are given in Fig. 4. The non-linear, first-harmonic intensity and amplitude ratios measured for the three bound fatty acid spin labels with $H_1 \sim 0.5$ G and magnetic field modulation frequencies of both 100 and 25 kHz, in the presence and absence of 7.5 mM $K_3Fe(CN)_6$, are given in Table 3. The corresponding relaxation enhancements, $\Delta(1/T_1)$, determined from the calibrations of ref. [11] or from

Table 3
Spin-lattice relaxation enhancements by ferricyanide, $\Delta(1/T_1)$, for 5-SASL, 12-SASL and 16-SASL non-covalently bound to BSA, determined from the intensity, ρ'_1, and amplitude, $\rho'_1(M_I)$, ratios of the out-of-phase and in-phase first-harmonic EPR spectra at modulation frequencies, ω_m, of 100 and 25 kHz[a]

Spin labels	Medium	ω_m (kHz)	$\rho'_1(M_I = +1)$	$\Delta(1/T_1)\times10^{-6}$ (s^{-1})	$\rho'_1(M_I = 0)$	$\Delta(1/T_1)\times10^{-6}$ (s^{-1})	ρ'_1	$\Delta(1/T_1)\times10^{-6}$ (s^{-1})
5-SASL	buffer	25	0.152		0.314		0.126	
	+7.5 mM $K_3Fe(CN)_6$		0.07	0.29	0.131	0.38	0.056	0.26
	buffer	100	0.19		–		0.173	
	+7.5 mM $K_3Fe(CN)_6$		0.119	0.33	–	–	0.118	0.33
12-SASL	buffer	25	0.192		0.333		0.12	
	+7.5 mM $K_3Fe(CN)_6$		0.088	0.26	0.144	0.37	0.06	0.23
	buffer	100	0.186		–		0.172	
	+7.5 mM $K_3Fe(CN)_6$		0.13	0.26	–		0.112	0.35
16-SASL	buffer	25	0.095		0.172		0.092	
	+7.5 mM $K_3Fe(CN)_6$		0.024	0.77	0.056	0.7	0.012	1.1
	buffer	100	0.142		–		0.148	
	+7.5 mM $K_3Fe(CN)_6$		0.0845	0.54	–	–	0.09	0.5

[a] T_1 calibrations are taken from ref. [11].

spectral simulations, are also given in Table 3. The values of $\Delta(1/T_1)$ determined for a given spin label from the different spectral parameters and at different modulation frequencies differ somewhat, but systematic trends between the different labels are largely preserved. In particular, the relaxation enhancements are uniformly greater for the 16-SASL position of labelling than for the other two labelling sites.

The second-order rate constants for spin exchange ($\langle k_{ex} \rangle$), calculated from the averaged values of $\Delta(1/T_1)$, are given for the three spin-labelled stearic acids in Table 4. These measured values are averaged over all occupied fatty acid binding sites, as indicated by the angular brackets. The corresponding values of k_{ex} for the free spin labels, which were determined from the exchange line broadening of the conventional EPR spectra in the absence of BSA, are also given in Table 4.

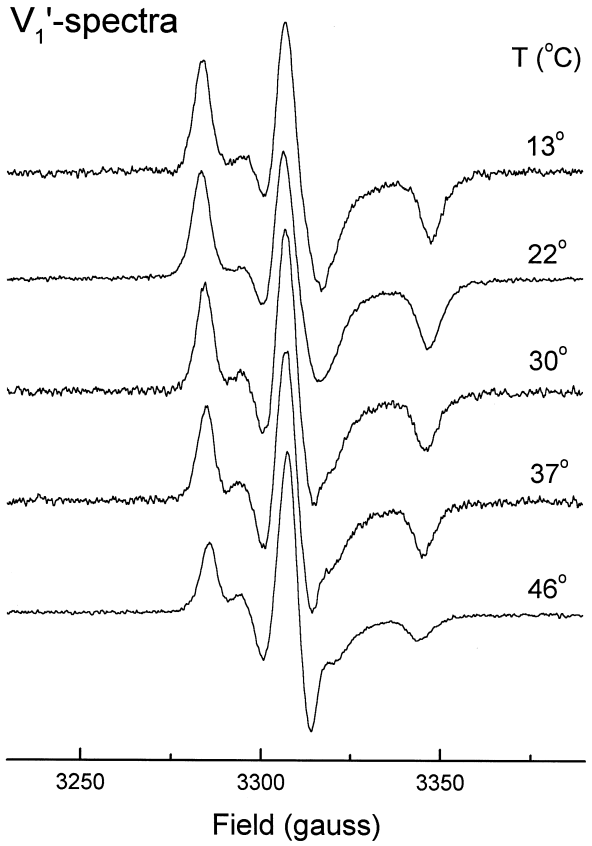


Fig. 5. Temperature dependence of the first-harmonic out-of-phase V'_1-EPR spectra of 12-SASL bound to BSA in phosphate buffer at pH 7.0. Modulation frequency 25 kHz and $H_1 = 0.5$ G.

Table 4
Second-order rate constants for Heisenberg spin exchange between ferricyanide and spin labels 5-SASL, 12-SASL, and 16-SASL free, k_{ex}(free), or bound, $\langle k_{ex} \rangle$, to BSA

Spin label	$\langle k_{ex} \rangle \times 10^{-9}$ [a] (M^{-1} s^{-1})	k_{ex}(free) $\times 10^{-9}$ [b] (M^{-1} s^{-1})
5-SASL	0.042 ± 0.01	0.41 ± 0.05
12-SASL	0.040 ± 0.01	0.61 ± 0.1
16-SASL	0.096 ± 0.015	0.8 ± 0.1

[a]For *n*-SASL bound to BSA.
[b]For *n*-SASL free in solution.

The temperature dependence of the relaxation enhancement caused by $K_3Fe(CN)_6$ was studied for 12-SASL bound to BSA. Out-of-phase, first-harmonic V'_1-EPR spectra recorded at different temperatures are given in Fig. 5. The EPR measurements of the intensity and amplitude out-of-phase/in-phase ratios were performed at a magnetic field modulation frequency of 25 kHz. In order to determine the values of $\Delta(1/T_1)$ from calibrations [11], the temperature dependence of the protein rotational correlation time (τ_R) was taken into account by using the Stokes–Einstein equation for τ_R and the temperature dependence of the viscosity of water (see Table 5).

4. Discussion

4.1. Accessibilities of the spin labels covalently and non-covalently bound to BSA

The essential parameter that defines the accessibility of the spin labels to the paramagnetic relaxation agent $K_3Fe(CN)_6$ in the aqueous phase is $\langle k_{ex} \rangle$, the mean effective rate constant, for the mutual spin exchange interaction (see Eq. 3). This quantity is directly related to the rate constant for bimolecular collision between the paramagnetic relaxant and spin label (e.g. ref. [19]). Two sets of reference data for the accessibilities of the spin-labelled fatty acids bound to BSA are the exchange rate constants for the spin labels free in solution and covalently bound to the single –SH group on BSA, respectively.

The values of k_{ex} for the covalently bound spin labels **I** and **II** are essentially the same, whereas that for spin label **III** which has a longer link to the point of attachment is twice as large (Table 1).

Table 5
Temperature dependence of the spin-lattice relaxation enhancements, $\Delta(1/T_1)$, rate constant for spin exchange with ferricyanide, $\langle k_{ex}\rangle$, and outer hyperfine maxima separation, $2A'_{zz}$(G), for 12-SASL bound to BSA, and of the rotational correlation, τ_R, calculated for BSA

T (°C)	$2A'_{zz}$ (G)	τ_R^a (ns)	$\Delta(1/T_1)\times10^{-6}$ [b] (s^{-1})	$\Delta(1/T_1)\times10^{-6}$ [c] (s^{-1})	$\langle k_{ex}\rangle\times10^{-9}$ (M^{-1} s^{-1})
13	64.3 ± 0.15	36	0.016	0.026	0.003
22	63.4 ± 0.15	30	0.26	0.23	0.033
30	62.2 ± 0.15	23.9	0.24	0.21	0.030
37	61.4 ± 0.15	20.9	0.29	0.25	0.036
46	60.15 ± 0.15	18	0.34	0.28	0.041

[a]Calculated for BSA at 22°C [3] using the Stokes–Einstein equation and the temperature dependence of the aqueous viscosity.
[b]Determined from the amplitude ratio, $\rho'_1(M_I)$.
[c]Determined from the double integrated intensity ratio, ρ'_1.

The value for the latter probably corresponds to a label close to the surface of the protein, as suggested by its rather mobile conventional EPR spectrum (Fig. 1). The exchange rate constants for all maleimide spin labels free in solution are much larger than those for the bound labels. This may be partly because of the higher diffusion rates of the free labels, in addition to increased accessibility.

Of the bound spin-labelled fatty acids, the values of $\langle k_{ex}\rangle$ for the 5-SASL and 12-SASL positional isomers are similar, whereas that for the 16-SASL positional isomer is approximately a factor of two larger (Table 4). For 16-SASL bound to BSA, the value of $\langle k_{ex}\rangle$ is similar to those for the more immobilised covalently linked labels **I** and **II**. The 5-SASL and 12-SASL positional isomers are bound to BSA in locations that are considerably less accessible to aqueous ferricyanide than are any of the covalently linked spin labels.

As expected, the free spin-labelled fatty acids have a much greater accessibility to $K_3Fe(CN)_6$ than do the bound fatty acids (see Table 4). The value of k_{ex} for 16-SASL in water is similar to that for the rather smaller free maleimide spin labels. For the aqueous 5-SASL and 12-SASL stearic and positional isomers, the values of k_{ex} are significantly lower than that for aqueous 16-SASL. A possible reason for this difference is an intrinsically higher segmental mobility of the terminal methyl end of the fatty acid, even in water. (Note that the small, uncharged maleimide spin labels **I**–**III** all have similar values of k_{ex} in water.) Interestingly, the variation in exchange rates between the different *n*-SASL fatty acid label positions, in water is in the same order as those for the bound fatty acids, i.e. 5-SASL ≥ 12-SASL > 16-SASL. This point is addressed further in a later subsection.

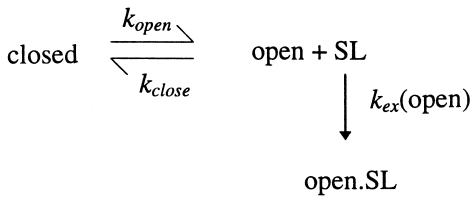
4.2. Fatty acid binding site

According to the crystal structure of the myristate complex with human serum albumin [6], the fatty acid molecules are located in long and narrow hydrophobic channels with the carboxyl groups exposed to polar residues at the surface of the protein. For subdomains IB and IIIB, the channels are ~17 Å in length, and are open at each end. Of the five bound myristates in the crystal structure, only for one is the electron density of the entire chain resolved. The last two or three methylene groups of the four remaining myristates were suggested to be disordered. The values of the exchange rate constants, $\langle k_{ex}\rangle$, show that the nitroxide group of 16-SASL which is attached near the terminal methyl group of the hydrophobic chain is even more accessible to water-soluble $K_3Fe(CN)_6$ than are those of 5-SASL or 12-SASL. A possible interpretation, consistent with the x-ray structure [6], is that in 16-SASL the nitroxide group protrudes somewhat from the hydrophobic channel and is located in a more polar environment with greater exposure to $K_3Fe(CN)_6$ than for 5- and 12-SASL. A rather similar conclusion was reached by Ge et al. [8] based on the increased rotational mobility of the bound 16-SASL relative to 5- and 12-SASL. A value of A_{zz} = 34.2 G, obtained for the principal hyperfine component of 16-SASL at −30°C is also consistent with a rather polar (although not aqueous) environment for this spin label bound to

protein [8]. Here we support these interpretations by using measurements that are directly sensitive to the location and accessibility of the spin label bound to the protein.

4.3. Kinetic model

An alternative kinetic scheme that takes into account the similar relative dependences of $\langle k_{ex} \rangle$ on the *n*-SASL spin-label position for bound and free fatty acids (particularly for 5-SASL and 16-SASL – see Table 4) in solution is given below. This consists of a rapid dynamic equilibrium of the fatty acid-bound protein between two conformations, 'closed' and 'open':



In the 'closed' state, spin exchange with the aqueous relaxation agent is assumed to be very low, consistent with the fatty acid being buried in the hydrophobic binding channel. Spin exchange takes place only in the 'open' state, where it is assumed to have characteristics comparable to those for exchange with the free spin label. If the occupation of the 'open' state is much smaller than that of the 'closed' state, i.e. $k_{close} \gg k_{open}$, then this model leads to the following simple expression for the effective spin exchange constant, $\langle k_{ex} \rangle_{eff}$:

$$\langle k_{ex} \rangle_{eff} = \frac{k_{open}}{k_{close}} \cdot k_{ex}(\text{open}) \quad (4)$$

where k_{ex}(open) is the intrinsic rate constant for spin exchange in the 'open' state. Eq. 4 is valid for multiple fatty acid binding to BSA, if the accessibilities of the various 'open' states are similar. It is seen from Eq. 4 that this model predicts that the spin-exchange rate constants measured for the different *n*-SASL will be in the ratio of the corresponding values of k_{ex}(open). The latter is assumed to display characteristics similar to those for the free spin label, and must take values $k_{ex}(\text{open}) \leq k_{ex}(\text{free})$. Taking the upper limit from the measurements with the free spin label yields values $k_{open}/k_{close} \sim 0.1$, 0.07 and 0.12 for 5-, 12- and 16-SASL, respectively. Free spin label at these relative concentrations in the presence of protein would be readily detectable in the conventional EPR spectra, which it is not (see Fig. 2). It therefore can be concluded that the values of k_{ex}(open) for the 'open' state are considerably smaller than those for the spin label free in solution, i.e. that the fatty acid still remains bound in the 'open' state.

The true situation is likely to be a hybrid of the two extremes, viz. the kinetic interpretation and the structural interpretation of the previous section. The increased value of k_{ex} for 16-SASL free in solution and the disordering of the terminal methyl end of the fatty acid chain found in the crystal structure probably both contribute to the accessibilities of the bound *n*-SASLs. In the latter connection, it should be noted that the *n*-SASL chains are longer than those of the bound myristates in the crystal structure and that the labelling position in 16-SASL lies beyond the terminal methyl group of myristic acid.

4.4. Temperature dependence

Evidence for a temperature-dependent conformational change in serum albumin has been advanced previously from an abrupt change, in the temperature interval 15–23°C, in slope of Arrhenius plots of the motional parameters of lipid spin labels bound to BSA [3]. It is seen from Table 5 that the spin exchange rate constant for 12-SASL bound to BSA is only weakly dependent on temperature in the range 20–46°C, but decreases sharply at lower temperatures between 13 and 22°C. This change in effective activation energy associated with the accessibilities of the spin-labelled fatty acids clearly reflects the same conformational change that was registered previously by the rotational mobility. In terms of the model with 'closed' and 'open' states given above, it represents a marked reduction in population of the 'open' configuration in the temperature regime below 20°C.

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