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BINDING OF NITROXIDE STEARATE SPIN LABELS TO BOVINE SERUM ALBUMIN

HANS HEINRICH RUF and MANFRED GRATZL

Department of Physiological Chemistry, University of the Saarland, 6650 Homburg/Saar (G.F.R.)

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SUMMARY

1. 12-Nitroxide stearate binds to bovine serum albumin at about four independent and equivalent binding sites with an association constant of about 10^6 M^{-1}. The binding at these high-affinity binding sites is significantly reduced by addition of unlabeled stearate. These data suggest that nitroxide stearates probe the high affinity binding sites for long-chain fatty acids.

2. Qualitative analyses of the ESR spectra of 5-, 12- and 16-nitroxide stearate bound to bovine serum albumin and measurements of the interaction of these compounds so bound with ferricyanide ion provide a rough description of the binding site as follows: the polar headgroup of the spin-labeled fatty acid is rigidly fixed, but fairly accessible to paramagnetic ions. The middle part of the hydrocarbon chain of bound stearate spin label also is rigidly fixed but differs in being shielded from the solvent, presumably by a hydrophobic cleft. The methyl terminus shows greater motion, appearing to move within a narrow cone, and also appears to be somewhat accessible to paramagnetic ions.

INTRODUCTION

Albumin binds a variety of small molecules and the transport of lipids in the blood is one of the basic functions of this protein. The binding of long-chain fatty acids to albumin has been investigated by various methods: equilibrium dialysis [1, 2], partition analysis [3–9] spectroscopic techniques [10, 11, 12], electrophoretic separation [13, 14], dielectric and viscosity measurements [13]. As a result of these studies albumin is believed to contain two or three high affinity binding sites and a large number of weaker binding sites for long-chain fatty acids [1, 3–5, 9]. An analysis of binding data in terms of multiple stepwise equilibria yielded a series of decreasing association constants [2, 7, 8].

Abbreviations: 5-nitroxide stearate, 2-(3-carboxypropyl)-2-tridecyl-4,4-dimethyl-3-oxazolidinyloxyl; 12-nitroxide stearate, 2-(10-carboxydecyl)-2-hexyl-4,4-dimethyl-3-oxazolidinyloxyl; 16-nitroxide stearate, 2-(14-carboxytetradecyl)-2-ethyl-4,4-dimethyl-3-oxazolidinyloxyl; TEMPO-OH, 4-hydroxy-2,2,6,6-tetramethylpiperidinooxyl; TEMPO-stearamide, 4-(octadecanoic acid amido)-2,2,6,6-tetramethyl-piperidinooxyl.

Although a large amount of thermodynamic data has been gathered, there is little information concerning the structure of the binding sites. This study has been undertaken to investigate the molecular properties of the binding sites for long-chain fatty acids, using spectroscopic probes. We have chosen a spin-label method using isomeric nitroxide stearates with the nitroxide radical located at various positions on the hydrocarbon chain of the fatty acid in order to probe the environment at different parts of the bound stearate molecule. While this work was in progress, binding of fatty acid spin labels to albumin was reported from other laboratories [16, 17, 18]. However, these studies did not clearly elucidate the nature and the structure of the binding sites involved.

The present study reports quantitative binding parameters for 12-nitroxide stearate, which indicate that the nitroxide stearates indeed probe the high-affinity binding sites at bovine serum albumin. In addition we contribute information on the nature of this binding site through qualitative analysis of the spectra of bound 5-, 12- and 16-nitroxide stearates and by measurement of the magnetic interaction of external ferricyanide ions with these spin labels.

MATERIALS AND METHODS

50 mM sodium phosphate buffer (pH 7.4) was used throughout in this study. Bovine serum albumin (crystallized) was obtained from Serva, Heidelberg, G.F.R. This albumin contained less than 15 μg of fatty acid/g of protein as checked in our laboratory by gas chromatography. L-Thyroxine sodium salt was purchased from Merck, Darmstadt, G.F.R., and the spin labels from Syva, Palo Alto, Calif., U.S.A. All other chemicals were of the purest quality available from Merck, Darmstadt, G.F.R.

ESR spectra were recorded with a Varian E-9 spectrometer at about 9.5 GHz. For the room temperature studies an aqueous flat cell (Varian E-248) was used. The measurements at liquid nitrogen temperature were carried out in quartz sample tubes having a 3 mm inner diameter in a cold finger dewar. The ESR spectrometer was connected to a Data General Nova 820 computer, which allowed double integration and superposition of ESR spectra.

The spin labels were added as methanolic solutions, the methanol concentration never exceeded 1.5% in the measured samples. Recording of a spectrum was started 1 minute after the addition of the probe. Spin label concentrations were determined by double integration and comparison with standard solutions of TEMPO-OH measured under identical conditions.

Since quantitative ESR has many pitfalls [28], we describe the evaluation of the concentrations of free or bound spin labels from the measured amplitudes a and b (Fig. 1B) in detail.

Under identical conditions (lineshape, spectrometer settings, adjustment of the aqueous cell in the cavity, solvent etc.) the concentration of a free radical is proportional to an amplitude of its ESR spectrum (normalized by the spectrometer gain: $a' = a/\text{gain}$, $b' = b' = b/\text{gain}$); hence

$$c_a = f_a \cdot a' \text{ and } c_b = f_b \cdot b'$$

Each factor consists of two terms, a "sensitivity" and a "lineshape" term, which are considered separately. Since the actual sensitivity of an ESR spectrometer depends on the polarity of the sample (affected both by the solvent and the protein concentration) and the adjustment of the flat cell in the cavity, we used the double integrals from the known amount of spin label to determine the actual sensitivity for each series of measurements. The lineshape term relates an amplitude of an ESR signal to its double integral. Thus it must be determined for each spin label in each physical state as follows.

(a) From a titration of spin label into buffer, a' versus c_{SL} (concentration of added spin label) is plotted. Because of dimerisation of nitroxide stearates this plot represents a saturation curve which is linear up to $c_{SL} = 3 \cdot 10^{-5}$ M. The slope of this linear part is taken as the reciprocal factor $1/f'_a$. The determination of f'_a was performed once for each spin label. The variation of the sensitivity from (a) to (b) (the titration of albumin) was taken into account by comparing the double integrals from the same amount of spin label under both conditions (I_a, I_b), where the different lineshapes are compensated by the integration procedure. Then $f_a = f'_a \cdot I_a/I_b$.

(b) From the titration of albumin with the spin label b' versus c_{SL} is plotted in a range, where the free spin label is less than 1 % of the added spin label. In this range the plot is found to be linear, and its slope yielded $1/f_b$. The double integral I_b was determined for the normalization of f'_a (see a).

As c_{SL} was also determined by double integration against a suitable spin standard, the error limits of the accuracy of the whole procedure are estimated to be ± 30 %, whereas its precision is much better (S.E.M. ≤ 5 %). Thus this method seems suitable for the study of the interaction or competition of various other ligands in the fatty acid binding of albumin.

RESULTS

Determination of binding parameters

The ESR spectra of nitroxide spin labels are sensitive to their molecular motion, the polarity of the environment and magnetic interactions of the spin label [19]. Therefore the binding of nitroxide stearates to macromolecules, accompanied by an immobilization of the nitroxide, can give information about the extent of binding as well as the microenvironment at the binding site.

The ESR spectrum of 12-nitroxide stearate at low concentrations in buffer (Fig. 1A) shows three sharp lines, indicating a fairly rapid isotropic motion of the molecule as a monomer. At nitroxide stearate concentrations above $5 \cdot 10^{-5}$ M, the amplitude increase is no longer proportional to the amount of spin label added and concomitantly a broad line of nearly 20 Gauss begins to appear.

This broad line indicates spin exchange between adjacent spin labels [20], due to the formation of nitroxide stearate dimers or oligomers. The formation of oligomers complicates binding studies of fatty acids [8]. We have found, that the concentration of monomeric nitroxide stearates never exceeds $1.5 \cdot 10^{-4}$ M at 22 °C, even at the spin-label concentration of $5 \cdot 10^{-3}$ M. Fortunately, our evaluation of bound and free spin-label concentration, is not influenced by the presence of oligomers.

When bovine serum albumin is added the spectrum of 12-nitroxide stearate changes to that shown in Fig. 1B which occurs as a superposed broad signal along

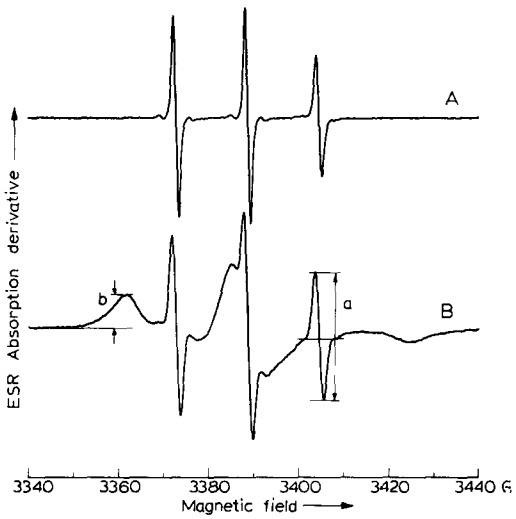

Fig. 1. ESR spectra of the 12-nitroxide stearate spin label (at 22 °C, microwave frequency 9.56 GHz, power 1 mW). (A) Spin label (5.4 μM) in buffer (modulation amplitude 0.4 G, gain 1250) (B) Spin label (54 μM) with bovine serum albumin (6 mg/ml) (modulation amplitude 2 G, gain 500) The evaluation procedure for the binding plots is explained in the text.

with the original three-line signal of the free spin label. This altered signal indicates immobilization of 12-nitroxide stearate as a consequence of binding to bovine serum albumin. In contrast to the broadened spectra obtained from oligomers, this type of broad signal represents monomeric spin labels with restricted motion. The spectral parameters *a* and *b* (Fig. 1B), showing the least interference between the spectra of free and bound nitroxide stearates, were used to determine the concentration of bound and free ligand as described under Methods.

Fig. 2a shows a logarithmic plot of the binding of 12-nitroxide stearate to bovine serum albumin and its competition with unlabeled stearate. It represents a simple titration curve which allows an estimate of the number of binding sites. The two curves, showing the effect of stearate on the binding of 12-nitroxide stearate, diverge progressively at higher concentration of the spin label. When the data in the absence of stearate are plotted according to Scatchard [21] (Fig. 2b), a straight line is obtained. The parameters of this line indicate that there are about four equivalent and independent binding sites for 12-nitroxide stearate with assosiation constants of $1 \cdot 10^6$ M^{-1}. Crude titrations of albumin with 5- or 16-nitroxide stearate yield approximately the same magnitude of binding parameters. Therefore it is concluded that all these nitroxide stearates share the same binding sites. If unlabeled stearate is present during binding of 12-nitroxide stearate a reduced binding of the latter is observed, leading to straight lines which are nearly parallel to the orginal one. From the binding parameters and the competition with stearate it is concluded that the nitroxide stearates bind to the high-affinity sites for long-chain fatty acids of bovine serum albumin.

The corresponding methyl ester of the spin labels and TEMPO-stearamide also bind to bovine serum albumin, but their solubilities in the aqueous buffer were

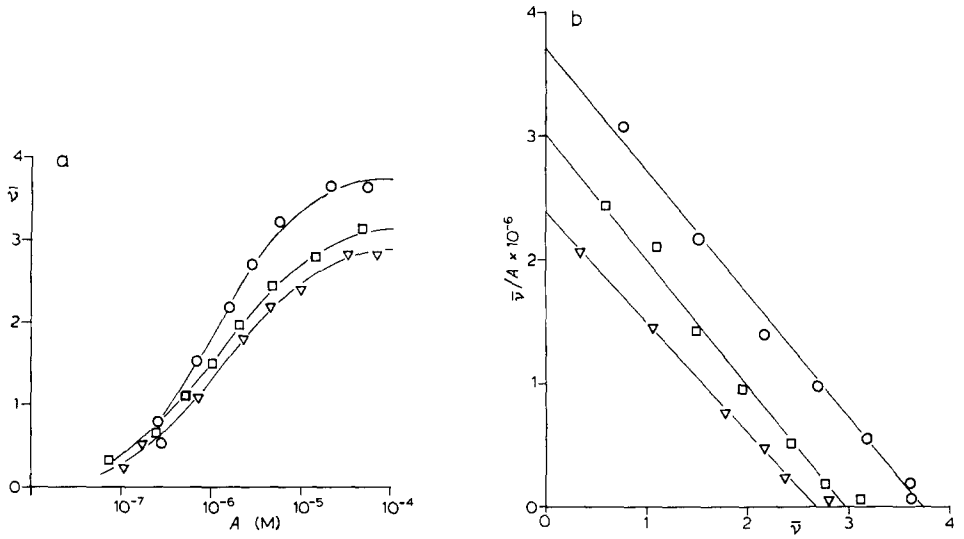

Fig. 2. Logarithmic (a) and Scatchard (b) plot for binding of 12-nitroxide stearate to bovine serum albumin and the influence of unlabeled stearate (A, concentration of free spin label; $\bar{\nu}$, mol bound spin label/mole albumin). Bovine serum albumin (6 mg/ml) was preincubated with the stearate for five minutes and then titrated with the 12-nitroxide stearate (at 22 °C, microwave frequency 9.56 GHz, power 1 mW, modulation amplitude 2 G: the gain was adjusted for equal amplitude of the spectra). ○—○, no stearate; □—□, 10^{-5} M stearate; ▽—▽, 10^{-4} M stearate.

so low, that the broad signals indicating dimer or polymer formation were observed below 10^{-6} M of spin labels. These broad signals make unfeasible the determination of binding parameters for the nitroxide stearate methylesters.

An interaction of thyroxin with the long-chain fatty acid binding of albumin has been reported [22]. The addition of L-thyroxine (up to about 1 mg/ml) to samples in which about half the binding sites are occupied by 12-nitroxide stearate, increases the amplitude of the free spin label signal by less than 0.5% of the total amount of spin label. Neither a change in the amplitude nor in the shape of the signal from the bound spin label could be detected. Thus an interaction between thyroxine and nitroxide stearate binding sites cannot be found.

Nature of the binding sites

The ESR spectra (Fig. 3) of the nitroxide stearates can provide information on the binding sites for long-chain fatty acids in bovine serum albumin. The spectra of the different nitroxide stearates show a strong immobilisation, but a different residual motion is expressed in the lineshapes for the different spin labels. For the bound 5-nitroxide stearate, a correlation time estimated according to ref. 23 was found to correspond to the motion of the albumin molecule as whole. Thus the nitroxide moiety of this spin label seems to be rigidly attached to the protein.

The bound 12-nitroxide stearate exhibits the same type of signal as the 5-nitroxide stearate, but the outer extrema are sharper and their splitting is decreased. This may indicate a small increase in motion and/or a less polar environment surrounding the nitroxide group in the 12-position.

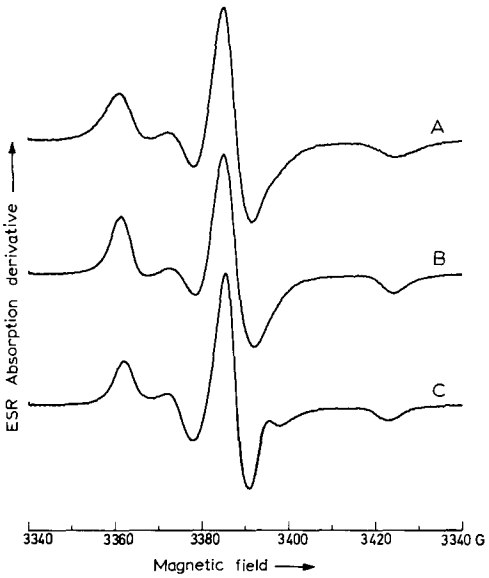

Fig. 3. ESR spectra of different nitroxide stearate spin labels (80 μM) bound to bovine serum albumin (60 mg/ml): (A) with 5-nitroxide stearate; (B) with 12-nitroxide stearate; (C) with 16-nitroxide stearate. (The ESR parameters are the same as in Fig. 2).

The 16-nitroxide stearate shows the type of signal indicating greater freedom of motion, of a fairly rapid, anisotropic type with an order parameter [24] of about 0.8. The estimated isotropic hyperfine splitting is about 16 G, representing a polar environment like that of water.

The dipolar interaction between a nitroxide radical and a paramagnetic ion in the aqueous solution can be used to probe the accessibility of the bound nitroxides for the ion [26]. Therefore this effect is useful in estimating whether the nitroxide groups of the different stearate spin labels are exposed to the solvent or not. Leigh [25] has shown that an enhanced relaxation due to the dipolar interaction between rigidly oriented spins causes a decrease in amplitude with almost no broadening of the ESR signal. Since in frozen samples the rigid lattice condition is fulfilled, we measure the influence of the paramagnetic ferricyanide ion on the ESR amplitude of the nitroxide stearates bound to albumin at 77 K.

When ferricyanide up to 0.1 M is added to albumin with about two binding sites occupied by nitroxide stearate, neither an increase of the free, nor a decrease of the bound spin label can be seen in the ESR spectra at room temperature. Thus ferricyanide does not affect the binding of the nitroxide spin labels and the decrease observed at 77 K represents the dipolar interaction with the bound nitroxide stearates (Fig. 4).

All spin labels used show a decrease in ESR amplitude with increasing concentrations of ferricyanide. The observed amplitude decrease falls off with a high power of the distance between nitroxide and ferricyanide [25]. The distance above which no decrease can be detected, has been estimated to be 6–8 Å [26]. So we can easily determine relative accessibilities within this range, whereas distances can be roughly estimated only.

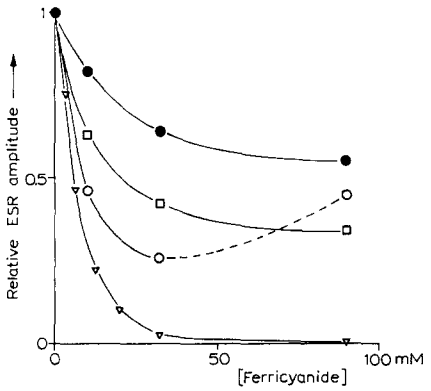

Fig. 4. Effect of ferricyanide on the ESR amplitude of free TEMPO-OH and different nitroxide stearates bound to bovine serum albumin (▽—▽, TEMPO-OH; ○—○-5-, -●—●-12- and □—□ 16-nitroxide stearate). Bovine serum albumin (6 mg/ml) was preincubated with spin label (30 μM). After addition of varying amounts of 1 M $K_3Fe(CN)_6$ the samples were frozen and measured in liquid nitrogen (at 77 °K, microwave power 20 μW, modulation amplitude 2.5 G). The relative amplitudes A/A_0 were corrected for dilution and plotted versus the concentration of ferricyanide.

The TEMPO-OH spin label which is water soluble and does not bind to albumin shows the strongest relaxation, indicating a close contact between ferricyanide and the free spin label. The nitroxide stearates are not suitable controls for demonstrating interactions with a free spin label, because freezing and thawing of the samples leads to oligomerization with accompanying broad signals even at low spin-label concentrations. Therefore TEMPO-OH was used as a probe which is accessible to the paramagnetic ion.

The bound nitroxide stearates exhibit only minor amplitude decreases compared to the TEMPO-OH. The albumin appears to shield the bound nitroxide stearates against close contact with ferricyanide. A distance of about 5 Å separating the ferricyanide from the nitroxide can be derived from an amplitude decrease of 50% according to [25, 26].

The 12-nitroxide stearate which exhibits the smallest decrease in amplitude must be buried most deeply in the albumin, whereas the nitroxide group attached closer to either end of the hydrocarbon chain in stearate shows greater exposure to the solvent. The increase in amplitude observed for 5-nitroxide stearate at the highest ferricyanide concentration tested remains unexplainable.

DISCUSSION

From the binding parameters and the competition with unlabeled stearate it is concluded that the nitroxide stearates bind as monomers to bovine serum albumin at high-affinity binding sites for long-chain fatty acids. The binding constants of about 10^6 M^{-1} for the nitroxide stearates is in good agreement with the binding constants previously reported for long-chain fatty acids [1, 3–5, 9]. In contrast to the results obtained by others with unlabeled fatty acids, the signal amplitude of the monomeric bound nitroxide stearates shows a distinct saturation at about 4 binding sites. Although from small variations of the spectra of bound 16-nitroxide stearate [17] and

TEMPO-stearamide [18] two types of binding sites have been proposed, neither our signals nor our binding plots provide evidence for low-affinity binding of monomeric nitroxide stearates. Binding of dimer or polymer nitroxide stearates cannot be excluded since the ESR signal of such bound oligomers is exchange-broadened and cannot be separated from the signal of oligomers in solution.

An alternative explanation for the saturation here reported might be that the amphiphilic properties of the fatty acids changed by the introduction of the nitroxide group. If this is so, the nitroxide stearates might not induce the slight unfolding of the albumin structure, already documented during the binding of fatty acids [13] and dyes [27]. The exposure of weaker binding sites for the interaction with additional ligands may be dependent on such unfolding.

The interference of unlabeled stearate with labeled stearate during binding cannot be interpreted in classical terms of competitive and noncompetitive inhibition because one of the ligands, as shown in this study, binds to four independent and equivalent sites and the other does not. Nevertheless the binding of 12-nitroxide stearate was found to be greatly diminished by the addition of stearate and it is concluded that spin-labeled stearic acids share the so called high-affinity binding sites of long-chain fatty acids. The lack of interference of L-thyroxine with the binding of 12-nitroxide stearate might be an indication that this hormone, although slightly diminishing fatty acid binding, does not interact with the high-affinity sites.

The qualitative interpretation of the ESR spectra of bound nitroxide stearates and the interaction with ferricyanide of the bound nitroxide stearates yields a rough portrait of the binding site which is thought to provide polar and hydrophobic interactions. The polar interaction has a considerably larger range than the hydrophobic interaction and may thus guide the carboxyl group of the fatty acid to its point of fixation. This would enhance the chance of alignment and close contact between the hydrocarbon chain of the ligand and a suitable nonpolar moiety on the protein necessary for the hydrophobic interaction. The polar "point of fixation" alone does not seem to be sufficient to explain the low mobility of either the nitroxide a C-5 of stearate or the corresponding methyl ester. Even the dipole moment of the ester presumably provides enough polar interaction for a fixation of the esters similar to that of the carboxylate anion. This interpretation is supported by recent studies on the binding of spin-labeled palmitic acid, which have shown that the low mobility at the carboxyl group is unaffected by esterification [16]. The strong interaction of bound 5-nitroxide stearate with ferricyanide implies that the region of the binding site interacting with the carboxy end of the fatty acid is fairly exposed to the aqueous environment. Furthermore, if the TEMPO-stearamide spin label binds to the same high-affinity binding site, the fairly large isotropic hyperfine splitting of its ESR signal [22] indicates that the polarity around the nitroxide and the polar headgroup is similar to that of water.

Hydrophobic interactions contribute to the rigid fixation of the hydrocarbon chain up to C-12 which is demonstrated by the low mobility of the 12-nitroxide stearate and the least accessibility of the bound nitroxide for the paramagnetic ferricyanide. At C-16 higher mobility is observed, although the motion is restricted to a narrow cone (order parameter about 0.8) and the methyl terminus becomes more exposed to the solvent. Spectra obtained from bound stearic acid derivatives bearing the nitroxide group at different distances from the carboxyl moiety also indicate a

nonmonotonic increase in motion from the polar COOH terminus to the nonpolar hydrocarbon tail [16]. Compared to the high fluidity of fatty acid chains in the hydrophobic region of membranes [24], the restricted mobility of stearic acid spin labels bound to albumin is remarkable and leads to suggest a narrow groove as the hydrophobic binding moiety which shields especially the 12-nitroxide stearate against interaction with ferricyanide.

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