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EPR study of the hydrophobic interaction of spectrin with fatty acids

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The hydrophobic interaction between spin-labelled stearic acid and spectrin was studied by electron paramagnetic resonance (EPR) and fluorescence quenching. The results are quantitatively interpreted in terms of two types of binding site on spectrin. A comparison between the results of the EPR and fluorescence experiments show the drawback of the fluorescence method in binding studies.

Introduction

The discoid shape of the human erythrocyte is believed to be primarily maintained by the underlying cytoskeleton which is composed mainly of spectrin [1]. The interaction of the membrane with spectrin has been the subject of extensive research and recently the role of electrostatic forces in this interaction have been discussed [2]. Nevertheless the existence of hydrophobic regions in the spectrin molecule [3] and in the lipid bilayer portions of the erythrocyte membrane suggest that hydrophobic interactions may also be relevant to the maintenance of erythrocyte shape. Interactions of this sort have been studied by a variety of methods and recently attempts have been made to directly examine the interaction between spectrin and phospholipids by measuring the attendant quenching of fluorescence of the spectrin molecule [3]. In these studies there is no way of correlating the degree of quenching with the number of bound molecules, since different binding sites will be associated with different degrees of quenching. The present report contains an account of the hydrophobic interaction between spin-labelled stearic acid and spectrin, as studied by electron paramagnetic resonance (EPR) and by fluorescence quenching. The interaction was studied by following (a) the changes in the EPR spectrum of spin-labelled stearic acid on addition of spectrin and (b) the changes in the intrinsic spectrin fluorescence due to addition of spin-labelled stearic acid. The results are quantitatively interpreted in terms of two classes of binding site. A comparison between the results of fluorescence and EPR measurements expose the drawback of the use of fluorescence in this type of study.

Methods and Materials

Spectrin extraction and purification

Erythrocyte ghosts were prepared according to the procedure of Dodge et al. [4]. Spectrin dimers were extracted from those ghosts according to the procedure of Marchesi [5] with some modifications by incubation at 37 °C for 30 min in the presence of 0.1 mM EDTA, 0.1 mM sodium phosphate buffer (pH 8) containing 0.12 mM phenylmethylsulfonyl fluoride (PMSF). The extract was concentrated by centrifugation in Amicon CF 25 cones and chromatographed on a Sepharose 4B column (50 × 1 cm) equilibrated with 25 mM Tris-HCl (pH 7.6) containing 0.1 M NaCl/5 mM EDTA/0.12 mM PMSF (Dherm et al. [6] and Ungewickell et al. [7]). The second elution peak which consists of spectrin dimers was collected and concentrated with an ultrafiltration cell and an Amicon YM 100 membrane. The purified spectrin was kept at 4 °C for the EPR and fluorescence experiments. Purity of the concentrated spectrin was tested in SDS-polyacrylamide gel according to Laemmli [8]. Protein concentration was esti-

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mated by the absorption at 280 nm taking the specific absorption of 1% protein at 280 nm as 10.7 [9].

EPR measurements

Fatty acid spin-label probe 5-DSA or 16-DSA (Sigma) was dissolved in methanol at a concentration of 4.7 mM. 5 μl of the probe were dried in a conic tube under nitrogen stream and then 1 ml of 25 mM Tris-HCl (pH 7.6) containing 0.1 M NaCl/5 mM EDTA/0.12 mM PMSF (Solution A) was added and the tube incubated in a shaking bath at room temperature for 90 min. This period of time was found to be necessary in order to get a stable EPR signal. Samples of 20 μl were further diluted with 100 μl Solution A and EPR spectra recorded on a Varian E-4 spectrometer. (Sweeptime, 8 min, field sweep 100 G, modulation amplitude 1 G, time constant 3 s.)

In order to study the effect of spectrin on the observed EPR spectra, increasing amounts of spectrin were added to 20 μl duplicate samples of the spin probe and diluted in Solution A to a final volume of 120 μl. The mean value of the height of the low-field signal in each duplicate was taken in arbitrary units.

The experiments with the unlabeled stearic acid were performed in a similar way to those for spin labeled stearic acid: 10 μl of stearic acid (Sigma) at a concentration of 4.7 mM in methanol was dried and diluted with Solution A. Increasing amounts of diluted unlabeled stearic acid were added to the spin-labeled stearic acid/spectrin mixture and the volume was adjusted to 120 μl with Solution A before recording the EPR spectra.

Fluorescence measurements

Spectrin dimers were diluted in Solution A to give a final concentration of 0.03 μM. Fluorescence measurements were performed with a Shimadzu RF-540 spectrofluorimeter, excitation wavelength 280 nm, slit width 2 nm.

Increasing amounts of the spin label probes 5-DSA or 16-DSA dissolved in ethanol at a concentration of 9.4 mM were added to the 0.03 μM spectrin sample and the fluorescence emission spectra was measured between 300 and 400 nm. The fluorescence spectrum of the spin label probes were taken for each sample and the difference spectrum recorded. The fluorescence emission intensity at 340 nm was recorded in arbitrary units.

Results

Addition of spectrin to solutions of spin-labelled stearic acid results in a decrease in the intensity of the EPR spectrum. The decrease is proportional to the concentration of spectrin up to a certain concentration, above which the intensity of the spectrum remains approximately constant (Fig. 1). When unlabelled stearic acid is added to solutions containing both spectrin and spin-labelled stearic acid the EPR signal is partially restored to its original value in the absence of spectrin. The increase in intensity depends on the ratio of unlabelled to labelled stearic acid and the concentration of spectrin (Fig. 2).

The spectrin–stearic acid interaction was also examined using fluorescence measurements. The addition of spin-labelled stearic acid to spectrin results in a quenching of the tryptophan fluorescence of the protein (Fig. 3).

Discussion

The initial sharp fall in intensity of the EPR signal of spin-labelled stearic acid on addition of spectrin can in principle be due to either chemical or physical

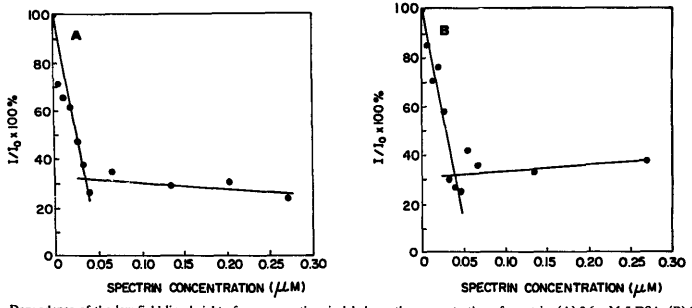

Fig. 1. Dependence of the low-field line height of paramagnetic spin labels on the concentration of spectrin: (A) 3.6 μM 5-DSA; (B) 3.6 μM 16-DSA. I_0 and I, EPR low-field line height of the spin label in the absence and in the presence of spectrin, respectively.

causes. However, there are two strong reasons for discounting a chemical reaction. If the fall in intensity is due for example to the reduction of the nitroxyl group by spectrin then this reaction should go to completion at high spectrin concentrations. This does not happen. Furthermore, addition of unlabelled stearic acid results in a partial restoration of intensity an observation which can be explained by the model described below, which includes only physical factors (Fig. 4). The key to the interpretation of the experimental results, and the basis of the model, is the fact that spin-labelled stearic acid gives a very broad EPR spectrum when bound to macromolecules in such a way that the motion of the nitroxyl group is strongly restricted. The steep initial fall in intensity can be explained by the binding of spin-labelled stearic acid to

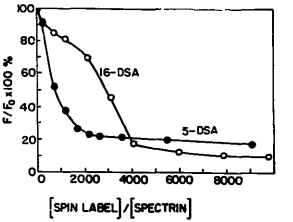

Fig. 3. Effect of paramagnetic spin labels 5-DSA and 16-DSA on fluorescence of spectrin: spectrin samples were dissolved in 25 mM Tris-HCl (pH 7.6) containing 0.1 M NaCl/5 mM EDTA 0.12 M PMSF to a final concentration of 0.3 μM. Paramagnetic spin label samples up to 0.3 mM were added to the spectrin sample. F_0 and F, fluorescence intensities in the absence and the presence of spin labels, respectively.

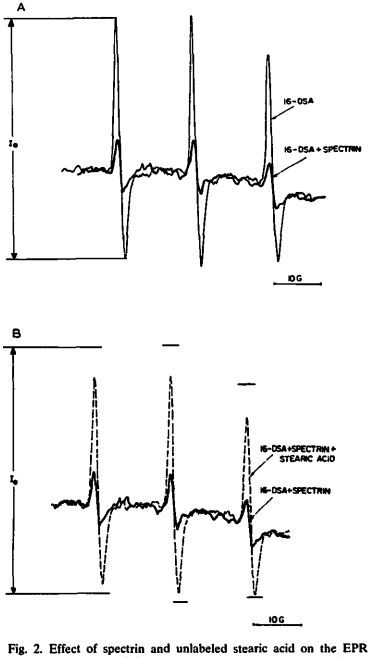

Fig. 2. Effect of spectrin and unlabeled stearic acid on the EPR spectra of 16-DSA: (A) 3.6 μM 16-DSA in solution $\pm$0.09 μM spectrin; (B) 3.6 μM 16 DSA + 0.09 μM spectrin $\pm$ 9 μM unlabeled stearic acid. I_0, Low field line height in the absence of spectrin.

spectrin. The fact that the plot of intensity against spectrin concentration is, within experimental error, a straight line suggests that the binding of spin-label is very strong. If there were only one kind of binding site then at large spectrin concentrations the sharp EPR line, characteristic of spin-label in solution, would vanish. The basic assumption of the model used to explain the present results is that the persistent sharp EPR spectrum is not due to free label in solution but due to bound label situated at sites at which the motion of the group is comparitively unrestricted. Two *classes* of adsorption site are thus postulated site 1 at which the label is strongly immobilized and site 2 at which the label is weakly immobilized. Let there be n_1 individual sites in site 1 and n_2 in site 2. Then the relative populations, p_1 and p_2, of the two sites at equilibrium are given by a Boltzmann-type distribution:

$$\frac{p_2}{p_1} = \frac{n_2}{n_1} e^{-\Delta G/RT} \qquad (1)$$

where ΔG is the difference in binding free energy at the two sites. *Provided that ΔG is not affected by cooperative effects*, p_2/p_1 is completely independent of spectrin concentration. At all concentrations of spectrin the numbers of bound spin-label at sites 1 and 2 are in constant ratio. When sufficient spectrin is present the spin-label is completely absorbed (the breakpoint, Fig. 4) and further addition of spectrin can neither give more binding, nor affect p_1/p_1. (However, see below.) The spin-label at site 2 gives the constant sharp EPR spectrum.

The addition of unlabelled stearic acid results in a competition for binding sites and releases part of the bound label. The spin-labelled stearic acid released from site 1 will result in an increase in the intensity of

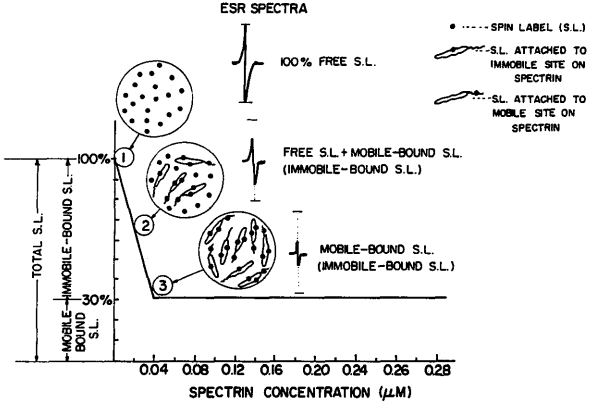

Fig. 4. Schematic model for stearic acid spin label binding to spectrin: (1) Free spin label in solution, the low field line height is proportional to the concentration of the free spin label in solution; (2) Spectrin is added in a small amount which does not bind all the free spin-label in solution. The EPR signal is reduced due to the binding of spin-label to the immobile sites of spectrin. Free spin-label and spin-label bound to mobile sites contribute to the observed spectrum. (3) No free spin-label remains in solution and the EPR signal is solely from spin-label attached to mobile sites on spectrin.

the EPR spectrum. Spin-label released from site 2 does not result in any significant change in the EPR spectrum because free spin-label and spin-label bound at site 2 give effectively the same spectrum. This can only be true if the motion of the spin-label bound at site 2 is comparatively unrestricted. Since the spectrin dimer has a molecular weight of nearly half-a-million daltons it is not immediately apparent why absorbed stearic acid should be motionally unrestricted. One possible explanation is that stearic acid is only bound through the carboxyl group at site 2 and that the hydrocarbon chain is not bound. This is very unlikely, there is no reason why a lengthy hydrocarbon chain should not be subject to strong dispersion forces, binding it to the spectrin dimer whether there is a hydrophobic neighbourhood to the carbonyl binding site or not. An alternative explanation is that site 2 is situated on a mobile part of the spectrin molecule. Support for the latter model might be provided by the work of Gratzer et al. on the NMR spectrum of spectrin [10] which consists of a very broad featureless absorption on which sits an area of highly resolved lines. These lines have been assigned mainly to hydrophobic amino acid residues. It is thus possible that the spectrin molecule contains one or more regions of highly flexible hydrophobic peptide chain. Spin-labelled stearic acid adsorbed onto these mobile regions would share the motional freedom of the peptides and could exhibit motionally narrowed EPR spectra. It should be noted that Fung et al. [11], although accepting a model that included flexible regions, suggest that the NMR spectra are best interpreted in terms of similar amino acid composition for the rigid and flexible parts of the protein.

At the breakpoint (Figs. 1 and 4) no free label remains in solution and the remaining EPR signal is proportional to the amount of bound spin-label on mobile sites, and the drop in intensity from the original spectrum is proportional to the amount of spin-label on immobile sites. It is thus possible to use the experimental concentrations of spectrin and spin-label to estimate the number of bound spin-labels at each kind of site on a single spectrin molecule. The data shown in Fig. 1 lead to estimated values of approximately 70 and 30 for sites 1 and 2, respectively. If the binding free energies of the spin-label do not vary greatly between different sites and ΔG can be put at zero in Eqn. 1, then the numbers of bound spin-labels are equal to the numbers of the two kinds of sites. However, if, as seems likely, the binding to the sides is of a very different nature, it is not possible to assume that ΔG is close to zero and p_2/p_1 cannot be estimated.

At the breakpoint the sites are still heavily populated, but as further spectrin is added to the solution

the spin-labels will tend to spread out over all the available spectrin molecules and the population *per spectrin molecule* will fall progressively. If there are cooperative effects on the conformation and/or binding energies of the adsorption sites changes in population could result in changes in ΔG and consequently in the ratio p_1/p_1 and the intensity of the EPR spectrum. This is a conceivable explanation for the slight rise in intensity after the breakpoint for the experiments with 16-DSA (Fig. 1B).

It is evident from the present EPR results that the binding of 5-DSA and 16-DSA to spectrin is very similar, as would be expected. Nevertheless there is a small but significant difference between the two spin-labels. The ratio of heights of the central and low-field lines differ in the mobile spectrum of the two spin-labels at high spectrin concentration (not shown). For 5-DSA the ratio is 1.52, compared to 1.42 in an aqueous solution of the spin-label. For 16-DSA the ratio is effectively the same, ~ 1.17, in the presence of absence of spectrin. Thus binding of 5-DSA at the 'mobile' site of spectrin seems to, relatively, restrict the motion of the nitroxyl groups more than is the case for 16-DSA. This suggests that the hydrocarbon chain is not strongly bound at this site and that the spin-label may be bound primarily via the carboxyl group. It is interesting in this connection that we have observed in our fluorescence experiments that 16-DSA induces a small concentration dependent shift in spectrin emission spectrum from 340 nM to 343 nM but 5-DSA induces a much larger shift from 240 nM to 353 nM. This could indicate that that part of the spin-label that quenches the fluorescence is bound by the carboxyl group and for such molecules the nitroxyl group of the 16-DSA is on the average further from the spectrin fluorophore. On the basis of these results in the present study some preference can be given to the supposition that spin-label at the mobile site is bound by the carboxyl group only. The fact that the EPR results for 5-DSA and 16-DSA are almost identical underlines the fact that the difference in the fluorescence results is not due to a difference in binding between the two labels.

It should be noted that the fluorescence experiments were carried out with constant spectrin concentration and varying spin-label concentration, while in the EPR experiments the spin-label concentration was constant. The fluorescence quenching approaches a plateau for a ratio of approx. 2000 5-DSA or approx. 4000 15-DSA molecules per spectrin molecule. This contrasts with a ratio of approx. 100 obtained in the EPR experiments, for saturation of all the sites of spectrin. It is thus possible that quenching is partially collisional and that spin-label molecules not directly adjacent to the spectrin fluorophores also contribute to quenching.

Future experiments with defined fragments of spectrin may enlighten our understanding as to the location of spectrin regions involved in the two types of interaction with fatty acids.

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