TOPOLOGY OF A PROTEIN SPIN LABEL IN ERYTHROCYTE MEMBRANES

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1. Introduction

Spin label electron paramagnetic resonance (EPR) is a useful means of studying reporter group sensitivity to the mobility and chemical environment of its host molecule [1]. In an attempt to understand the erythrocyte membranes on a molecular level, spin label EPR methods have been used to monitor motional and structural behavior of membrane proteins and lipids. In particular, maleimide-analogue sulfhydryl spin labels have been used to study the protein components of both normal [2–7] and abnormal [8] erythrocyte membranes. Conventional EPR spectra provide information about molecular motion in the fast time range of 10^{-11}–10^{-7}s. [2–6,7] while saturation transfer EPR monitors relatively slower motions in the range of 10^{-7}–10^{-3}s. [7]. Therefore, the combination of these two techniques allows us to detect a wide range of molecular events occurring in intact membranes. Up to the present time, however, the spin label binding sites have not been well characterized, and it is therefore difficult to associate spectral information with specific protein molecules in membranes.

We have prepared spin-labelled right side out (RSO) vesicles both in the presence and in the absence of ascorbic acid, a chemical which reduces radicals to make spin labels EPR-silent. Since erythrocyte membranes are impermeable to ascorbic acid, we have selectively placed ascorbic acid molecules either inside or outside the vesicles and are therefore able to monitor the signals which arise from either the inner or from the outer surface of the vesicles. We find that ~ 80% of the spin-label intensity arises from label sites at the inner membrane surface. We have also isolated the cytoplasmic peripheral proteins, the spectrin–actin complex, from spin-labelled membranes and have demonstrated that ~ 75% of the spin labels are attached to this complex. Thus the principal component for spin-labelled erythrocyte membranes can be associated with the spectrin–actin complex, and the spin-label spectra can therefore be used as a spectroscopic probe to study the spectrin–actin complex in intact erythrocyte membranes.

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2. Materials and methods

2.1. *Sample preparation*

Membrane ghosts were prepared by the methods in [9]. Briefly, fresh blood cells were pelleted by centrifugation and then washed 3 times with phosphate saline buffer. The cells were lysed using 40 vol. 5 mM sodium phosphate buffer at pH 8 and washed 3 times to give hemoglobin-free white membrane ghosts [10].

RSO vesicles were prepared by the method in [11]. Membranes from 2 ml packed cells were brought to 5 ml total vol. with 20 mOsm phosphate buffer at pH 7.6. This diluted sample was then passed through a 26 g needle 4 times to give RSO vesicles, which were then centrifuged at 22 000 × *g* for 10 min. Vesicles were stored in vesicle storage buffer solution (20 mM KCl and 0.5 mM NaN_3 in 0.7 mM phosphate buffer. at pH 7.6). One batch of fresh RSO vesicles was made up with 5 mM ascorbic acid in the buffer solution in order to trap ascorbic acid inside RSO vesicles. Ascorbic acid was also added to membrane sample to serve as control.

Spectrin–actin complex was prepared by the

methods in [11]. White membrane ghosts were incubated at 37°C in low ionic strength buffer (0.3 mM phosphate buffer at pH 7.6) to solubilize spectrin–actin. The insoluble components were removed by centrifugation. The spectrin–actin solution was concentrated to ~ 3 mg/ml by ultrafiltration. The spin-labelled spectrin–actin complex was prepared similarly from the spin–labelled membrane ghosts (see below for labelling method). Sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis (by the methods in [12]) was performed on all samples. The gels of spin-labelled samples as presented in fig.1 show good agreement with those of unlabelled samples as well as those in [11], indicating that spectrin–actin can be extracted from spin–labelled membrane samples as easily as from unlabelled membranes.

Fig.1. SDS–polyacrylamide gel electrophoresis by the methods in [12] of spin-labelled samples: (A) membrane sample; (B) low ionic strength extract spectrin–actin complex; (C) insoluble residue of (B). Protein concentrations of these samples were different. EPR experiments were performed on these samples to give results presented in table 2.

2.2. *EPR sample preparation*

Immediately after preparation, samples of RSO vesicles with and without ascorbic acid inside were labelled with *N*-(1-oxyl-2,2,6,6-tetramethyl 4-piperidinyl) maleimide (Mal-6) (30–50 μg/mg protein) in the dark for 1 h. Membrane samples with and without ascorbic acid were also labelled similarly. All samples were then washed with the proper buffers to remove excess spin labels. After the third wash, EPR spectra were taken. Washing a particular sample was terminated when its EPR spectrum was the same as the one taken after the previous wash. A portion of the spin-labelled RSO vesicles was incubated for 2 min at room temperature in 5 mM ascorbic acid and was then washed to remove excess ascorbic acid. Unsealed spin-labelled membrane sample was also incubated with ascorbic acid as a control in parallel with the vesicle sample.

All preparations for each run were done at 4°C on the same day.

2.3. *RSO sideness assay*

The glyceraldehyde 3-phosphate (G 3-P) dehydrogenase accessibility assay was used to determine the percentage of RSO vesicles in each sample. The procedure was that in [9] with minor variations. Samples consisting 10 μg Mb protein in 0.1 ml were preincubated for 1 min with an equal volume of 5 mM phosphate buffer at pH 8 either with or without 0.2% Triton X-100. Sodium pyrophosphate (30 mM) adjusted to pH 8.4 just before use, was added to give 1 ml sample vol. followed by the addition of 0.03 ml 4 M sodium arsenate and 0.05 ml 20 mM nicotinamide adenine dinucleotide. After the addition of 0.1 ml 15 mM G 3-P at pH 7, the reaction was followed spectrophotometrically (340 nm) for 75 s. The increase in absorbance between 15 and 75 s was taken as a measure of enzyme activity. Percentage of RSO vesicles was determined from the absorbance readings for the reactions in phosphate buffer with and without Triton X-100 buffer. The assay was carried out at room temperature on fresh (prepared on the same day) vesicles with and without ascorbic acid either inside or outside of the vesicles.

2.4. *Protein determination*

Protein concentrations were determined by using a simplified Lowry method [13]. Generally, protein concentration was determined on the EPR sample after the experiment was performed.

2.5. *EPR measurement*

The EPR measurements were carried out on a Varian E109E X-Band spectrometer at 20°C [7] immediately after the samples were prepared. Signal intensities were obtained directly by double integration of the spectra with a Nicolet 535 time averager which was interfaced with the spectrometer. Spectral baselines were carefully adjusted to minimize integration error [14]. A spin label sample with known concentration was used for calibrating spin label concentrations from signal intensity data.

3. Results and discussion

The EPR spectra of erythrocyte Mb that are labelled with Mal 6 for 1 h in the dark at 4°C exhibit strongly (~ 90%) and weakly (~ 10%) immobilized components, similar to those in [4]. The spin label concentration is ~ 25 nmol labelled SH/mg protein (~ 15×10^{-18} mol SH/cell, assuming 6×10^{-10} mg protein/cell), which is in good agreement with work done under similar conditions [3]. Spin-labelled samples of RSO vesicles give EPR spectra indistinguishable from those of spin-labelled membranes. The spectra of RSO vesicles with ascorbic acid are similar to those of RSO vesicles in line shape but differ substantially in integrated signal intensity. Control samples of unsealed membranes which were treated with ascorbic acid in a fashion similar to the vesicle samples give no EPR signal. These results indicate that under our conditions, ascorbic acid effectively reduces all of the accessible spin labels. The total signal intensity, percentage of RSO vesicles, and total protein concentration of all samples were determined and the values obtained from a typical run are presented in table 1. The signal intensity of the membrane, or vesicle, sample was normalized to per milligram of protein. This value is then used to account for the contribution of EPR signals arising from the small amount of unsealed membranes in the vesicle samples. Finally the signal intensity of vesicles in each vesicle sample with ascorbic acid either inside or outside was used to calculate signal intensity per milligram of vesicle protein not accessible to ascorbic acid. These values are also presented in table 1.

The results from RSO vesicle samples with ascorbic acid outside (from 4 different blood samples) indicate that 80 ± 5% of the total EPR signal originates from spin labels attached to the SH groups (and possibly a few amino groups) which are located at the inner surface of the RSO vesicles, and are inaccessible to ascorbic acid reduction. The other 20 ± 5% signal is reduced by ascorbic acid in the external medium. RSO vesicle samples with ascorbic acid trapped inside the vesicles yield an EPR signal reduced to 10 ± 2% of the intensity in the absence of ascorbic acid, indicating that ~ 10% of the labels are located at the outer membrane surface. An understanding of the remaining

Table 1
EPR signal intensities of Mal-6 spin-labelled erythrocyte membrane vesicles treated with ascorbic acid from a typical experimental run

	Signal intensity (arbitrary unit)	% RSO vesicles	Total protein conc. (mg)	Signal intensity/ mg vesicle protein[a]
A	86.8	84.5	5.05	17.2
B	53.0	74.2	4.93	14.5
C	19.0	86.1	4.75	1.9

[a] See text for definition

A, sample of spin-labelled RSO vesicles
B, sample of spin-labelled RSO vesicles with ascorbic acid outside
C, sample of spin-labelled RSO vesicles with ascorbic acid inside

10% signal intensity which appears to be reduced by both procedures may depend on a better understanding of the molecular events occurring in the samples, such as the extent to which ascorbic acid penetrates the erythrocyte membrane bilayer, and the interaction between ascorbic acid and membrane proteins as well as the lateral diffusion of these molecules in membranes. However, membranes prepared with slightly different procedures may already introduce some variation of the fine structure of the membranes which may affect the results, and a more quantitative description may therefore not be worthwhile.

In addition to determining the location of the signal sites, we further extracted the cytoplasmic peripheral proteins, the spectrin–actin complex, from membranes in low ionic strength medium and found, as shown in table 2, that ~ 75% of the total EPR signal arises from this complex, in good agreement with the vesicle studies.

Erythrocyte membranes contain several kinds of proteins, and spin labelling the proteins in intact membranes by an alkylating reagent such as Mal-6 might be assumed to give a heterogeneous system of labelled sulhydryl groups (and may be a small percentage of other nucleophilic groups) of all proteins, with EPR and saturation transfer EPR spectra of these samples providing only general information about the protein system. Therefore, most of the previous EPR studies have suggested wide-spread protein involvement in their studies [2–6,8]. However, our studies indicate that ~ 80% of the labelled sites are on the cytoplasmic (inner) surface of the intact erythrocyte membranes, and that ~ 75% of the total labelled sites belong to the spectrin–actin complex. Other studies using impermeable SH reagents have also indicated that only a small amount (~ 3%) of the erythrocyte membrane SH groups reside at the exofacial membrane surface [15]. Early *N*-ethyl maleimide (NEM) studies indicated that 50% of the total NEM was associated with the spectrin–actin complex [16]. Although Mal-6 is a derivative of NEM, the two molecules are stereochemically quite different from each other and we would expect the two compounds to have only qualitatively, but not quantitatively similar reactivity with the erythrocyte membranes. Furthermore, in the earlier NEM studies, membranes were incubated with NEM at 37°C [16] and in our studies membranes were incubated with Mal-6 at 4°C. Both the lower temperature and the larger size of the Mal-6 molecule lead us to expect that Mal-6 would interact less with the relatively buried SH groups. Thus, under our labelling conditions, we have selectively labelled relatively more of the spectrin–actin complex. This finding allows us to reexamine some of the previous EPR studies and suggest that, for example, the calcium and pH effects reported [2,6] are primarily related to the spectrin–actin complex as reported [17]. The spectral changes which were observed may be related only to specific changes in the spectrin–actin complex and are not necessarily related to wide-spread lipid mobility and molecular packing.

Table 2
EPR signal intensity of membranes and low ionic strength extract spectrin–actin complex

	Signal intensity/mg protein
Membranes	44 ± 1
Spectrin–actin complex	33 ± 1

The present studies do not allow us to comment in detail upon the microenvironment and chemical nature of the labelled sites. However, we suggest that studies involving specific modifications of the spectrin–actin network may provide useful information about these label sites in the future. At the same time, the present studies provide a unique spectroscopic probe for monitoring the general molecular properties of the spectrin–actin complex in intact membranes [7] and exploring under various conditions, the molecular arrangement and dynamics of these proteins, which are believed to control the biconcave shape of the erythrocyte [18,19].

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