ROTATIONAL DYNAMICS OF SPECTRIN IN SOLUTION AND ANKYRIN BOUND IN HUMAN ERYTHROCYTE MEMBRANE

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1. Introduction

Some essential properties of the red cell membrane include its high resistance and deformability, the maintenance of the red cell shape and the uniform distribution of transmembrane proteins. They arise from the presence of a cytoskeleton associated with the cytoplasmic surface of the membrane. It is mainly composed of spectrin, actin and of the protein 4.1 (nomenclature of Steck [1]).

The interactions between the above components and the details of their binding with the erythrocyte membrane have been the object of many investigations [2–4]. Conventional electron paramagnetic resonance (EPR) measurements performed on spin-labeled spectrin have given some insights on the temperature and Ca^{2+}-dependent changes in conformation of spectrin [5]. In this work we report the application of saturation transfer (ST) EPR spectroscopy to the study of spectrin. It is a recently established method which allows the study of the slow rotational movements of proteins in the time range of 10^{-3} to 10^{-6} s [6]. A report of this technique applied to spectrin-actin has already appeared [7]. However, these experiments have been performed at pH 4.5 on a protein precipitate, and are not directly relevant to physiological conditions. We have found that ST EPR spectra of spin-labeled spectrin dimers and tetramers in solution or reassociated to ankyrin on the membrane were essentially the same. The rotational dynamics of spectrin measured by this technique are independent of its state of polymerisation as well as of its interaction with other membrane components. These results are discussed in the light of previous electron microscopic results on the shape of spectrin and in terms of spectrin-membrane interactions.

2. Materials and methods

Spectrin dimers and tetramers were prepared from freshly drawn human blood according to [8] and stored in 0.7 mM Na/Na_2PO_4, 20 mM KCl, 0.5 mM NaN_3, pH 7 buffer at 2°C. Spectrin concentration was calculated on a basis of MW 460 000 and $E^{1\%}_{280}$ = 10.1 [9,10]. The maleimide spin-labeled spectrin (MSL-spectrin) was prepared with 3-maleimido-2,2′,5,5′ tetramethyl-1-pyrrolidinyloxyl from Syva as in [5]. Spectrin-depleted inverted vesicles were prepared according to [11]. MSL-spectrin was reassociated with freshly prepared vesicles as in [11]. Special care was taken to wash the membranes until uncombined spectrin was eliminated. The purity of spectrin just as the quality of its binding to vesicles was controlled by sodium dodecyl sulfate polyacrylamide gel electrophoresis. Further controls were also made by velocity sedimentation on a Spinco analytical ultracentrifuge to assess the dimeric or tetrameric state of spectrin.

Conventional and ST EPR spectra were recorded with a Varian E-109 spectrometer on line with a Tracor NS 570 multichannel analyser for signal averaging. The samples were studied in 50 μl quartz cells set in a quartz Dewar for temperature control. First harmonic in phase spectra were recorded at 1G modulation (100 kHz) and second harmonic out of phase at 6G modulation (50 kHz). The ST EPR spectra were analyzed according to [6]. The microwave strength received by the sample was calibrated as described in [6] with peroxylamine disulfonate synthesized according to [12]. In order to avoid the possible autoaggregation of spectrin, measurements have been performed at protein concentration of about 4 mg/ml. In these conditions the signal/noise

ratio was small and imprecision in the ratio of peak intensities H''/H of the ST EPR spectra could not be avoided.

3. Results

3 molecules of MSL are bound (per 460 000 dalton unit) on both spectrin dimers and tetramers [5]. The conventional EPR spectrum of MSL-spectrin is mainly due to strongly immobilized probes (fig.1a). The ST EPR spectrum obtained on the same sample is shown in fig.1b. From the ratio of peak intensities L''/L and H''/H, the rotational correlation times τ_L and τ_H respectively can be deduced by comparison with the reference spectra of MSL hemoglobin [6]. The corresponding values obtained for the spectra shown in fig.1 are included in table 1 together with the estimate of τ_C measured in the central region of the spectra.

Below pH 6, spectrin abruptly precipitates [13]. The ST EPR spectrum recorded on spectrin dimers in 5 mM acetate buffer pH 4.5 is given in fig.1c. Finally one has shown in fig.1d the ST EPR spectrum of MSL-spectrin dimers reassociated at pH 7 with inverted erythrocyte vesicles previously devoided of their cytoskeleton by low ionic strength extraction. We obtained the same results with spectrin tetramers.

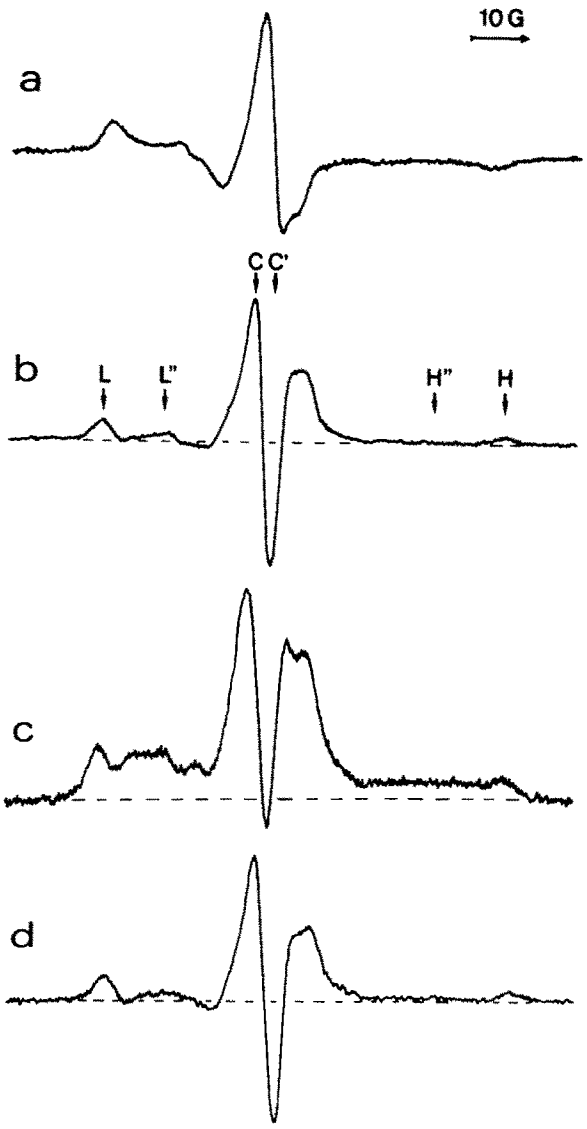

Fig.1. Conventional (a) and Saturation Transfert (b, c, d) EPR spectra of MSL-spectrin dimers at 2°C. Spectrin at pH 7 (a, b); at pH 4.5 (c); ankyrin-bound spectrin on membrane at pH 7 (d). The spectra obtained with MSL-spectrin tetramers are identical. Experimental conditions are given in the text.

Table 1
Rotational correlation time (τ_L, τ_C, τ_H) of MSL-spectrin dimers measured from ST EPR spectra of fig.1

	L''/L	τ_L sec	C'/C	τ_C sec	H''/H	τ_H sec
Spectrin, pH 7 in solution	0.28	6×10^{-6}	−0.98	10^{-7}	0.22	1×10^{-5}
Spectrin, pH 4.5 precipitated	0.84	2×10^{-4}	−0.14	4×10^{-6}	0.87	6×10^{-4}
Ankyrin-bound spectrin pH 7 on membrane	0.32	6×10^{-6}	−0.86	2×10^{-7}	0.29	2×10^{-5}

MSL-spectrin tetramers give identical values

4. Discussion

ST EPR spectroscopy has been usefully applied to a large number of biological systems [14] in spite of the difficulties encountered when the systems under study are undergoing anisotropic diffusion. In the latter cases the values of the correlation time τ_L, τ_C and τ_H measured at different field intensities by comparison with ST EPR reference spectra of isotropic MSL hemoglobin can differ widely [14]. This is also the case for spectrin (table 1). Good confidence in the values of τ_L and τ_H has however been demonstrated in some cases of anisotropic movement [15].

The conventional EPR spectrum of MSL spectrin (fig.1a) shows that almost all of the labels undergo slow rotational movements. This condition is necessary for ST EPR measurements and the correlation times one measures can be assigned to the rotation of the entire molecule. However we found no difference whatever (dimer or tetramer) was the state of polymerisation of spectrin. This result implies that the movements we observed reflect more surely either local rotational movements of part of the protein in the vicinity of the labels or the bending and flexing of the molecule. Similar hypotheses were proposed to interpret ST EPR spectra of monomeric actin included in F actin filaments [16].

Shotton et al. [17] have shown by electron microscopic studies that spectrin dimers after low-angle shadowing are elongated and very flexible molecules of 97 nm length. In order to adapt this size and to bend easily, they have proposed that spectrin would consist of several tertiary compact domains linked by flexible regions. This hypothesis is consistent with our findings on the mobile character of spectrin in solution as deduced from ST EPR spectra. Extension of ST EPR method on theoretical and experimental grounds for the analysis of anisotropic movements is in progress [14]. One can hope in the future to more quantitatively analyze the present data.

Fung et al. [7] have reported ST EPR measurements on spectrin-actin complex precipitated at pH 4.5. They found long rotational correlation time ($\simeq 10^{-3}$ s) as is, for example, the case for MSL-hemoglobin precipitated with ammonium sulfate [6]. This value is at the upper limit of resolution of the ST EPR method. Comparison in this case of the dynamics of spectrin in solution and membrane-bound can be hazardous and the contribution to the spectra of the motion of vesicles as a whole should be considered. We should notice that the values of $\tau_L \simeq 2 \times 10^{-4}$ s and $\tau_H \simeq 6 \times 10^{-4}$ s obtained in this work for purified spectrin dimers at pH 4.5 (table 1) are smaller than the one reported for spectrin-actin at the same pH [7]. This difference can reflect an increased immobilisation of spectrin in an actin network. These experiments performed in drastic pH conditions cannot be interpreted in terms of specific spectrin-membrane interactions.

Contrarily, in the conditions of this work spectrin is known to be strongly associated ($K_D = 5 \times 10^{-8}$ M) with a membrane protein called ankyrin [4,11]. Spectrin tetramers are presumably the functional entities present in erythrocytes [18]. They are formed by the 'head-to-head' association of two dimers [17]. When they are reassociated to vesicles the two constituting dimers are specifically bound to the membrane (Y. Lemaigre-Dubreuil, in preparation). We have found no evidence of slowing down in the movements of MSL probes of spectrin dimers and tetramers reassociated to membranes as compared to those of spectrin in solution. The spectra of fig.1b and 1d are very similar and give close values of rotational correlation time (table 1). One can infer that the conformation of spectrin is not strongly modified, for example constrained in a more globular structure, after binding ankyrin. Thus, although spectrin appears to be tightly bound to ankyrin through restricted areas of association, our ST EPR results suggest that the rest of the spectrin molecule interacts loosely with the cytoplasmic surface of the membrane. Further studies are necessary to evaluate whether this is also the case when spectrin tetramers are crosslinked with actin and protein 4.1 in the erythrocyte cytoskeleton.

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