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Rapid diffusion of spectrin bound to a lipid surface

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Abstract

Human erythrocyte spectrin was labelled with the probe 5,5′-disulfato-1-(6-hexanoic acid *N*-hydroxysuccinimide ester)-1′-ethyl-3,3,3′,3′-tetramethylindocarbocyanine (Cy3). Cy3-spectrin was bound to the outer surface of dimyristoylphosphatidylcholine (DMPC) multilamellar vesicles and its diffusion measured by fluorescence recovery after photobleaching (FRAP). It was found that at 30°C, above the lipid gel to liquid-crystalline phase transition of the lipids, Cy3-spectrin had an unexpectedly high diffusion coefficient $D = (2.1 \pm 0.6) \times 10^{-7}$ cm^2/s. At the phase transition, diffusion of Cy3-spectrin was only slightly lower; $D = (1.3 \pm 0.3) \times 10^{-7}$ cm^2/s, whereas at 14°C, well below the lipid phase transition, diffusion was found to be much slower with $D = (3.1 \pm 0.12) \times 10^{-9}$ cm^2/s. The fast diffusion of Cy3-spectrin on the lipid surface implies that the individual bonds which bind spectrin to the lipid surface must rapidly be made and broken. In the light of these results, spectrin–lipid interactions alone appear unlikely to have any significant role in supporting the cell membrane. Probably, the interactions serve only to localise the spectrin at the inner lipid surface in order to facilitate formation of the cytoskeleton.

Keywords: Erythrocyte; Fluorescence recovery after photobleaching; Cy3; Phosphatidylcholine; Fluorescent labeling; Cytoskeleton

1. Introduction

Spectrin is the predominant component of the erythrocyte cytoskeleton that adds strength to the cell membrane and helps maintain the erythrocyte shape. It is well documented that the erythrocyte cytoskeleton is bound to the erythrocyte membrane through interactions between both cytoskeletal proteins and transmembrane proteins [1,2]. Spectrin is coupled to the transmembrane protein, band 3, via ankyrin and to another transmembrane protein, glycophorin C via protein 4.1. Spectrin is also linked to vertices composed predominantly of actin oligomers, protein 4.1 and other accessory proteins. However, there is an increasing body of evidence that spectrin can also interact with the lipids on the inner membrane leaflet. The significance of such spectrin–lipid interactions is as yet unknown but many different roles have been postulated. Early studies suggested that spectrin–lipid interactions helped maintain the lipid asymmetry of the erythrocyte membrane but this is no longer thought to be the case since the role of an ATP-driven lipid translocase was eluci-

Abbreviations: Cy3, 5,5′-disulfato-1-(6-hexanoic acid *N*-hydroxysuccinimide ester)-1′-ethyl-3,3,3′,3′-tetramethylindocarbocyanine; DMPC, dimyristoylphosphatidylcholine; FRAP, fluorescence recovery after photobleaching; MLV, multilamellar vesicles; PS, phosphatidylserine

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dated [3–5]. Other possible roles for spectrin–lipid interactions include the further strengthening and shape regulation of the cell membrane or to direct and concentrate unbound spectrin molecules at the inner lipid leaflet to facilitate protein–protein interactions [6].

Erythroid spectrin is a dimer consisting of α and β subunits with molecular masses of 270 kDa [7] and 246 kDa [8], respectively. Spectrin dimers can form higher oligomers by head-to-head interactions, with tetramers being predominant within intact erythrocytes [9]. The α- and β-subunits of spectrin exhibit homology, both sharing a common 106-amino-acid repeat motif which forms a structurally identical barrel, composed of 3 α-helices [10–12]. The subunits interact along their length to form a coiled-coil like rod structure of ∼100 nm [12–15] that can compress and expand together.

The lipid specificity of spectrin–lipid interactions is still unclear since different studies have produced conflicting evidence. The majority of studies of spectrin–lipid interactions have shown that spectrin can interact with both neutral and negatively charged lipids. The binding affinity of spectrin for negatively charged lipids has in some studies been shown to be higher than that of spectrin–neutral lipid interactions [16–20]. Other studies, however, have shown spectrin affinities for both neutral and negatively charged lipids to be similar [21–23]. It was also suggested in some of the earlier studies that spectrin may insert into the lipid bilayers [16–18], but a more recent study found no evidence for this in bilayer vesicles [21]. Finally, there is one report that spectrin does not bind to neutral or negatively charged lipids with significant affinity [24].

Spectrin–lipid interaction studies have up to now focused on the type of interaction and the binding affinities of spectrin for various lipids and under varying conditions, but the mobility of spectrin on the lipid surfaces has not previously been investigated. Such studies could provide further insight into the nature of spectrin–lipid interactions. In this study, fluorescence recovery after photobleaching (FRAP) has been used to analyse the diffusion of Cy3-labelled spectrin bound to the outer lipid surface of DMPC multilamellar vesicles (MLVs) above and below the phase transition temperature of the lipids.

2. Materials and methods

2.1. Materials

Cy3 was purchased from Amersham (Cambridge Biosciences). DMPC was purchased from Sigma.

2.2. Purification of erythrocyte spectrin

Spectrin was purified from freshly drawn blood according to the method of Takeshita et al. [20], and its purity checked by 7.5% SDS–PAGE according to the method of Laemmli [25].

2.3. Cy3 labelling of spectrin

Cy3 labelling of spectrin has been previously described by Cherry et al. [26]. Briefly, spectrin was dialysed into 5 mM sodium phosphate (pH 7.5), and made to a final concentration of 1 mg/ml. One M sodium carbonate (pH 9.2) was added to a final concentration of 50 mM Na_2CO_3. 36.5 μg Cy3, dissolved in 125 μl distilled water, was added to 372 μg spectrin and incubated at 4°C for 40 min. Unconjugated dye was removed by PD10 size exclusion chromatography. The Cy3 concentration was calculated from the absorbance at 552 nm, using an extinction coefficient of 150 000 $M^{-1}\,cm^{-1}$. The Bradford assay was used to calculate the protein concentration. The dye to protein ratio was typically 10:1.

2.4. Manufacture of DMPC MLVs

5 mg DMPC (50 mg/ml in chloroform/methanol 3:1) was dried thinly onto the side of a round bottomed flask under argon. Two ml of buffer at 37°C was added to the round-bottomed flask and the lipids allowed to rehydrate at 37°C for 30 min with intermittent swirling. The vesicles were harvested and kept under argon at 4°C for up to 2 days.

2.5. Binding of Cy3-spectrin to MLVs

Cy3-labelled spectrin was dialysed into 100 mM NaCl, 10 mM sodium phosphate, 1 mM sodium azide, 1 mM EDTA, 0.2 mM DTT (pH 7.2) and concentrated to ∼1 mg/ml. Ten μl of MLVs were

pelleted at 5000 rpm and the pellet resuspended in a minimum amount of buffer followed by addition of 100 μl Cy3-spectrin, and incubated for 30 min at 30°C in the dark. Unbound spectrin was removed by pelleting the vesicles and resuspending the pellet in buffer; this process was repeated three times.

It is conceivable that some Cy3-spectrin could become entrapped within the vesicles if they open and reseal during the above procedure. To check for this possibility, 50 μl of MLVs were pelleted at 5000 rpm and the pellet resuspended in 200 μl calcein (50 μM), and incubated for 30 min at 30°C in the dark. The free calcein was then removed by pelleting the vesicles and resuspending the pellet in buffer; this process was repeated three times. The vesicles were then resuspended in 3 ml buffer and fluorescence measured on a Shimadzu RF5000 at 30°C with excitation wavelength set at 495 nm and emission scanned from 500 to 550 nm.

2.6. FRAP measurements

The FRAP system was essentially the same as described in a previous paper [27] but with the mechanical beam-splitter replaced by an electro-optic modulator [28] and the second fluorescence illumination light source, the air-cooled argon ion laser, replaced by a mercury lamp (Nikon, Model HB10101AF). The optical filter and dichroic mirrors described in the previous publications [27,28] were changed to ensure compatibility with the spectral properties of Cy3-spectrin. A small sample of vesicles, with Cy3-spectrin bound, were deposited on a poly-L-lysine-coated glass microscope slide and covered with a glass coverslip which was sealed with silicon grease. The slide was then placed onto the temperature-controlled microscope stage (Physitemp, Model TS-4) and allowed to equilibrate to the desired temperature prior to FRAP measurements. After equilibration, the 514 nm beam of an argon ion laser (Innova 100-10) was focused onto the desired area on the liposome or background. The laser beam was of Gaussian cross-sectional intensity, with a half-width at $1/e^2$ height of the laser beam at its point of focus equal to 1.24 μm (spot radius). FRAP measurements were recorded and analysed as previously described [28].

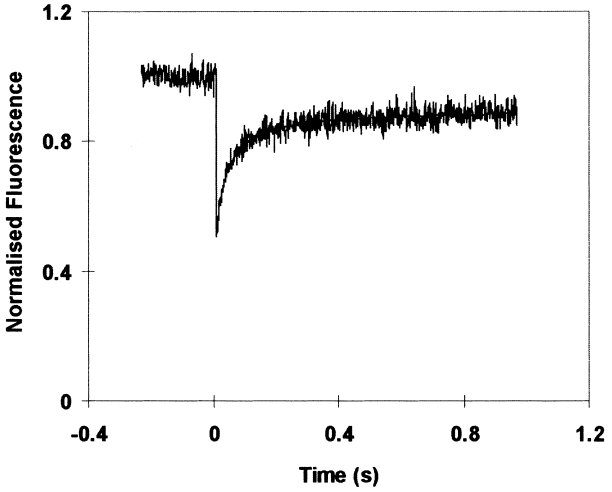

Fig. 1. FRAP curve of Cy3-labelled spectrin bound to the outer lipid surface of DMPC MLVs and measured at 30°C. The diffusion coefficient of Cy3-spectrin, fit from 30 individual summed curves, was calculated as $(2.1 \pm 0.6) \times 10^{-7}$ cm^2/s with 89% of the total fluorescence recovered.

3. Results and discussion

The diffusion of Cy3-spectrin bound to the outer surface of DMPC MLVs was studied at three different temperatures. The diffusion coefficient of Cy3-spectrin at 30°C, well above the gel to liquid crystalline phase transition of the lipid ($T_c = 23.7$°C) was found to be $(2.1 \pm 0.6) \times 10^{-7}$ cm^2/s with 89% of the

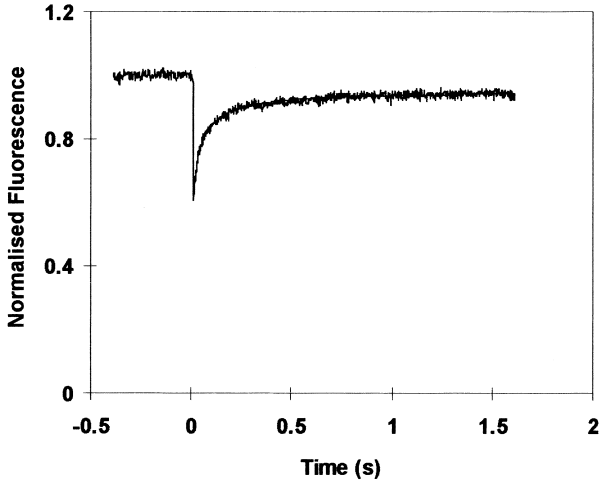

Fig. 2. FRAP curve of Cy3-labelled spectrin bound to the outer lipid surface of DMPC MLVs and measured at 22°C. The diffusion coefficient of Cy3-spectrin, fit from 30 individual summed curves, was calculated as $(1.28 \pm 0.3) \times 10^{-7}$ cm^2/s with 90% of the total fluorescence recovered.

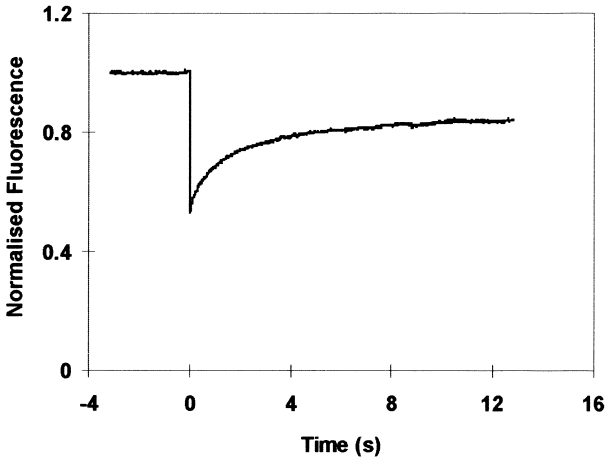

Fig. 3. FRAP curve of Cy3-labelled spectrin bound to the outer lipid surface of DMPC MLVs and measured at 14°C. The diffusion coefficient of Cy3-spectrin, fit from 30 individual summed curves, was calculated as $(3.1 \pm 0.1) \times 10^{-9}$ cm^2/s with 71% of the total fluorescence recovered.

fluorescence recovered after photobleaching (Fig. 1). At 22°C, the diffusion coefficient of Cy3-spectrin was found to be $(1.28 \pm 0.25) \times 10^{-7}$ cm^2/s with 90% of the fluorescence recovered after photobleaching (Fig. 2). Below the phase transition, at 14°C, the diffusion coefficient of Cy3-spectrin was found to be $(3.1 \pm 0.12) \times 10^{-9}$ cm^2/s with 71% of the fluorescence recovered after photobleaching (Fig. 3).

The diffusion of Cy3-spectrin at both 22°C and 30°C is surprisingly fast. The diffusion coefficient is similar to that of free Cy3-spectrin in solution, which was shown to be $(1.9 \pm 0.6) \times 10^{-7}$ cm^2/s at 30°C, $(3.4 \pm 1.0) \times 10^{-7}$ cm^2/s at 22°C and $(3.1 \pm 0.8) \times 10^{-7}$ cm^2/s at 14°C (Fig. 4), all with 100% fluorescence recovery. The fast diffusion of Cy3-spectrin bound to DMPC at 22°C and 30°C could be a result of several different factors. One possibility is that the Cy3-spectrin diffusion coefficient measured was that of residual free Cy3-spectrin in solution. It was clearly shown, however, that little free Cy3-spectrin was present in solution, as FRAP measurements of the background (i.e., away from any liposomes) showed much weaker fluorescence intensities and there was no recovery after photobleaching (Fig. 5). Another possibility is that Cy3-spectrin had become entrapped in the vesicles during the labelling and washing procedure. For this to happen the vesicles must break and reseal as it would be highly unlikely for Cy3-spectrin to permeate the lipid bilayer. If Cy3-spectrin did become entrapped within the vesicles during the labelling and washing procedure, it would also be expected that the Cy3-spectrin would be removed from inside the vesicles upon subsequent washing, resulting in little or no labelling. In addition, unlabelled DMPC MLVs incubated with calcein showed no detectable fluorescence after washing under identical conditions to those used to prepare vesicles for FRAP experiments. It was assumed that if the liposomes were breaking and resealing, the calcein molecules would easily become entrapped

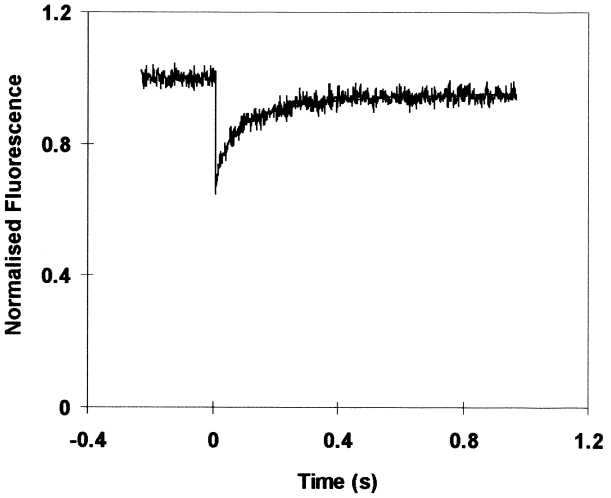

Fig. 4. FRAP curve of free Cy3-labelled spectrin in 100 mM NaCl, 10 mM sodium phosphate buffer (pH 7.4) at 14°C. The diffusion coefficient of free Cy3-spectrin, fit from 10 individual summed curves, was calculated as $(3.1 \pm 0.8) \times 10^{-7}$ cm^2/s with 100% of the total fluorescence recovered.

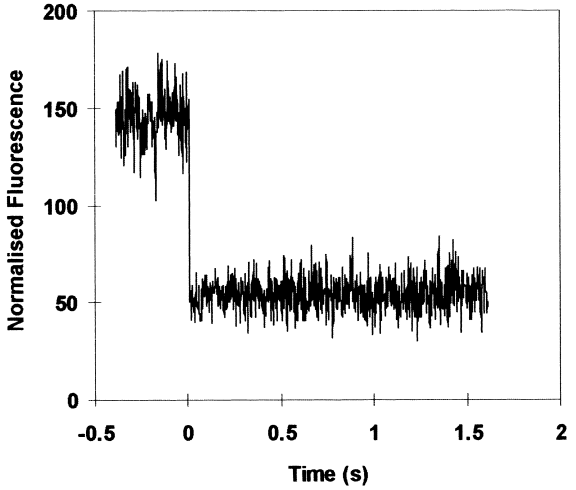

Fig. 5. FRAP curve of Cy3-spectrin deposited on the surface of a poly-L-lysine-coated slide at 30°C.

within the vesicles. As no evidence of calcein entrapment was found, it is highly unlikely that Cy3-spectrin could be entrapped. The decrease in Cy3-spectrin diffusion at 14°C when bound to DMPC is yet further evidence that the high diffusion coefficients are not due to free spectrin, since diffusion in solution exhibits little dependence on temperature.

It is highly unlikely that the fluorescence recovery is affected by polarised photobleaching followed by rotational diffusion [29,30]. Previous studies [31] have shown that spectrin flexibility results in almost complete randomisation of the probe within 10 μs, which is much faster than the recovery seen in these experiments.

The fast diffusion of Cy3-spectrin bound to DMPC can be explained by multiple weak spectrin–lipid interactions that are constantly being broken. Weak spectrin–lipid interactions do not necessarily imply that spectrin molecules readily dissociate from the lipid surface. Since spectrin has multiple sites of lipid interaction, the spectrin molecules may be able to diffuse rapidly over the surface as individual bonds are broken and reformed whilst the molecules remain bound to the lipid surface. For a spectrin molecule to dissociate, it would require the simultaneous dissociation of all its sites of lipid interaction. Thus this proposed mechanism is not in disagreement with previous studies which measure low dissociation constants for spectrin binding [16,17,19–21,23,32,33].

The observation of multiple weak interactions between spectrin and lipids is in agreement with Maksymiw et al. [19], who showed that the binding energy per mole of dimyristoylphosphatidylserine was less than thermal energy at room temperature, and Bitbol et al. [21], who postulated that spectrin interacted with phosphatidylserine (PS) molecules by a dipolar interaction of spectrin with the lipid–water interface. Such interactions would enable the strong association of spectrin with the lipid surface and also permit fast diffusion. A perturbation, however, of the spectrin–lipid interactions by the covalently bound fluorophores can not be entirely discounted.

The marked decrease in Cy3-spectrin diffusion below T_c could be a result of several different factors. Spectrin is known to expand upon decreasing temperature [34] which could result in different spectrin–lipid interactions that are stronger than those at 22°C or 30°C. MacDonald [32] showed that the apparent K_d of spectrin for binding to PS increases upon increasing temperature between 25°C and 37°C; however, it was also noted that the apparent maximum spectrin binding decreased with increasing temperature which suggested different types of lipid–spectrin interactions at different temperatures. It could therefore follow that at 14°C, spectrin forms more sites of lipid association that results in a slowing of spectrin diffusion on the lipid surface. The ordered lipid structure at 14°C could also favour stronger spectrin–lipid interactions as the lipids may be unable to break free of the spectrin interactions which are thought to occur at higher temperatures. Another possible reason for slower diffusion is insertion of hydrophobic domains of spectrin into the lipid bilayer of the vesicles, as suggested by Mombers et al. [16]. Spectrin has since been shown to have such hydrophobic domains in numerous studies [35–37]. Insertion into the lipid bilayer, however, seems unlikely as Maksymiw et al. [19] showed that spectrin does not significantly change the phase transition properties of DMPC bilayer vesicles, and other studies have revealed no evidence of spectrin insertion into lipid bilayers [21].

Any residual Cy3-spectrin which is not bound to the liposomes will bind to the polylysine coated slide where it is immobile (see Fig. 5). When the FRAP measurements are recorded from the vesicles it is likely that there will be a small contribution to the overall measurement by the out-of-focus plane fluorescence of polylysine-bound Cy3-spectrin. The immobile fractions seen on the vesicles can therefore be accounted for by the background fluorescence created by immobilised Cy3-spectrin lying underneath the vesicles which were also deposited onto the polylysine slide.

In conclusion, the present studies indicate that spectrin–lipid coupling is via multiple weak interactions that are rapidly broken and reformed. The fast diffusion rate suggests that spectrin–lipid interactions contribute little to the strength of the cell membrane. It should be noted that ankyrin binding to spectrin results in an increase in spectrin–lipid dissociation [22]. Once spectrin is complexed with the other members of the erythrocyte cytoskeleton, the total surface area of spectrin free to form spectrin–lipid interactions will be much decreased. In addition, spectrin

will be separated from the membrane by the linker proteins, ankyrin and protein 4.1. Therefore, the most likely role of such spectrin–lipid interactions is to localise and concentrate unbound spectrin molecules at the lipid surface in order to facilitate protein–protein associations involved in the formation of the erythrocyte cytoskeleton.

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