Interaction of Bovine Erythrocyte Spectrin with Aminophospholipid Liposomes

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Abstract. Interaction of bovine erythrocyte spectrin with aminophospholipid (phosphatidylethanolamine, phosphatidylserine and their mixture) vesicles was studied by means of intrinsic fluorescence quenching and fluorescence polarization of 1,6-diphenyl-1,3,5-hexatriene. Similarly as human and pig erythrocyte spectrin, bovine red blood cell spectrin interacts with vesicles prepared from these phospholipids. In model membranes, spectrin induced an increase of order parameter while in natural, red blood cell membranes spectrin binding was rather connected with a decrease in this parameter. The interaction of spectrin with the PE/PS vesicles was not affected by high concentrations of urea. These vesicles also did not protect spectrin from being denatured by urea.

Key words: Spectrin — Phospholipid bilayer — Fluorescence quenching — Erythrocyte membrane

Introduction

The red blood cell membrane skeleton is composed of several different proteins including spectrin, actin, band 4.1, band 4.9, adducin, tropomyosin and tropomodulin (for a review see Steck 1989; Bennett 1990). The membrane skeleton seems to be essential for maintaining the erythrocyte shape and the mechanical properties (Elgseater and Mikkelsen 1991). A major element of the membrane skeleton is spectrin, a water-soluble, high-molecular weight, tetrameric protein of elongated shape (200 nm long and $\sim$ 2 nm in diameter). Spectrin is composed of heterodimers made up of two non-identical subunits, α ($M_r \sim 280$ kDa) and β ($M_r \sim 247$ kDa). The primary structures of these polypeptides have been determined recently (Sahr

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et al. 1990; Winkelman et al. 1990). The maintenance of normal deformability appears to be essential for the erythrocyte function and survival. Spectrin is linked to the membrane bilayer mainly by ankyrin (high affinity binding site) which binds the cytoplasmic domain of the anion transporter protein (for a review see e.g. Steck 1989; Bennett 1990). It was also found to interact directly with membrane phospholipids either in the cells (Haest et al. 1978; Sikorski and Kuczek 1985) or in particular, in model systems (e.g. Juliano et al. 1971; Mombers et al. 1980; Sikorski et al. 1987; Bitbol et al. 1989; Michalak et al. 1990). The interaction of bovine erythrocyte spectrin with phospholipids has not yet been studied. In this paper we describe the interaction of spectrin from bovine erythrocytes with liposomes prepared from amino phospholipids. In particular, the study was focused on the effects of this interaction on properties of the protein and on the mobility of hydrocarbon chains of membrane phospholipids.

Materials and Methods

Spectrin from bovine erythrocytes was isolated according to the standard method (Sikorski et al. 1987) with a small modification according to Sikorski, Terlecki and Zembroń (unpublished). Briefly, erythrocytes were isolated from fresh bovine blood by centrifugation and washing in 310 mosmol phosphate buffer prepared by adjusting 0.103 mol/l Na_2HPO_4 to pH 7.4 with 0.155 mol/l NaH_2PO_4 (Dodge et al. 1963), frozen in liquid nitrogen in 15 ml aliquots and kept at −70 °C, for up to two months. To isolate spectrin, 2–3 aliquots of erythrocytes were thawed and diluted tenfold with 20 mosmol phosphate buffer (prepared by dilution of 310 mosmol phosphate buffer). Ghosts were obtained by centrifugation at $10,000 \times g$ for 30 min at 0–4 °C. After several washes with the same buffer and in the same conditions, pelleted ghosts were washed with two volumes of ice cold 0.3 mmol/l sodium phosphate, 0.1 mmol/l EDTA, pH 7.2 containing 5 μg/ml phenylmethylsulfonyl fluoride, by centrifugation at $40,000 \times g$ for 20 min. Spectrin dimers were extracted with one volume (8–12 ml) of the same solution for 30 min at 37 °C. After centrifugation for 40 min at $40,000 \times g$ (0–4 °C) the supernatant ($A_{280} \sim 1.1$–1.8) was chromatographed on a Sepharose CL 4B column (1.8 × 65 cm) equilibrated with 5 mmol/l sodium phosphate buffer (pH 7.2) containing 50 mmol/l NaCl, 0.1 mmol/l 2-mercaptoethanol, 0.1 mmol/l EDTA and 1 mmol/l NaN_3. In such conditions the spectrin dimer gave a major peak in the column profile. The purity of the isolated protein was tested by SDS polyacrylamide (7%) gel electrophoresis stained with Coomassie blue. In one experiment we were able to obtain 5–10 mg of pure spectrin dimer. Isolated in this way, spectrin did not exhibit any visible changes (e.g. increased turbidity or a tendency to precipitate) such as seen with stored spectrin preparations. Our preparations were active in binding ankyrin (Sikorski et al. prepared for publication). Isolated protein was dialysed overnight against the buffer used for the measurements: 0.1 mol/l Tris-phosphate buffer (pH 7.7) containing 50 mmol/l NaCl, 0.1 mmol/l EDTA, 0.1 mmol/l 2-mercaptoethanol.

Phospholipid (phosphatidylethanolamine and phosphatidylserine from bovine brain, Koch Light Laboratories) liposomes were prepared and fluorescence measurements were performed as described previously (Sikorski et al. 1987; Michalak et al. 1990). Briefly, chloroform was evaporated from phospholipid solution with the use of an oil vacuum pump

for 1–2 hours. The phospholipids were hydrated in 12.5 mmol/l sodium borate buffer, pH 8.0, for 40 min, shaken and sonicated for 20 min with a MSE sonicator and centrifuged at 12,500 × g for 20 min. The supernatant was used for experiments. Fluorescence measurements were carried out with the use of a Perkin-Elmer MPF 3L spectrofluorimeter equipped with a temperature controlling device. The excitation and emission wavelength was 290 and 337 nm respectively. F_0 was fluorescence of spectrin in the absence of phospholipid vesicles.

Labeling of membranes with 1,6-diphenyl-1,3,5-hexatriene (DPH) was performed by mixing DPH solution in tetrahydrofuran (2×10^{-3} mol/l) with sample buffer to obtain a final concentration of DPH of 2×10^{-6} mol/l. Equal volumes of DPH and membrane suspensions were mixed and incubated 1 hour before measurements were started. In polarization experiments, the probe fluorescence was excited at 360 nm and fluorescence emission was measured at 430 nm.

Protein concentration was determined according to Bradford (1976), and phospholipid phosphorus according to Bartlett (1959).

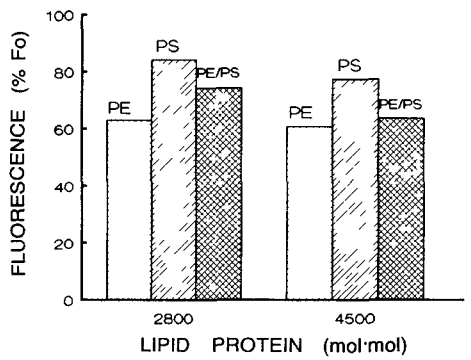

Figure 1. The effect of phospholipid liposomes on the intrinsic fluorescence of purified bovine red blood cell spectrin. PE, liposomes prepared from phosphatidylethanolamine; PS, liposomes prepared from phosphatidylserine; PE/PS, liposomes prepared from a mixture of phosphatidylethanolamine and phosphatidylserine (60 : 40). For other details see the text.

Results

Fig. 1 shows the effect of sonicated phospholipid vesicles on the intrinsic fluorescence of bovine red blood cell spectrin. Similarly as with human red cell spectrin (Sikorski et al. 1987), these vesicles induced quenching of 25% (PS) to 40 % (PE) of the intrinsic fluorescence of this protein. Liposomes prepared from a mixture of these phospholipids (PE:PS 60 : 40) quenched up to ~ 36% of spectrin fluorescence. This Figure gives examples of data for two protein:lipid molar ratios. Apparent values of K_D obtained in these conditions (according to Bagshaw and Harris 1987) were: PE vesicles, 9.2×10^{-5}; PS vesicles, 1.8×10^{-5}; and PE/PS vesicles, 3.5×10^{-5} mol/l. The above values may explain the controversy concerning the specificity of spectrin towards PS vesicles (Bitbol et al. 1989; Maksymiv et al. 1987). Some of the data suggested that spectrin has a preference for an interaction with PS

containing bi- and monolayers (Mombers et al. 1979; 1980; Maksymiv et al. 1987); others (Sikorski et al. 1987; Bitbol et al. 1989) did not observe this specificity. Our data may suggest that PS vesicles exhibit a higher affinity but a lower "capacity"; in the case of fluorescence quenching method, this not necessarily means that more PE vesicles are bound. The effect may just be stronger. Also the accessibility and the number of binding sites for each phospholipid in the spectrin molecule may be different. In a further experiment, we addressed the question whether spectrin affects the physical state of the membrane bilayer.

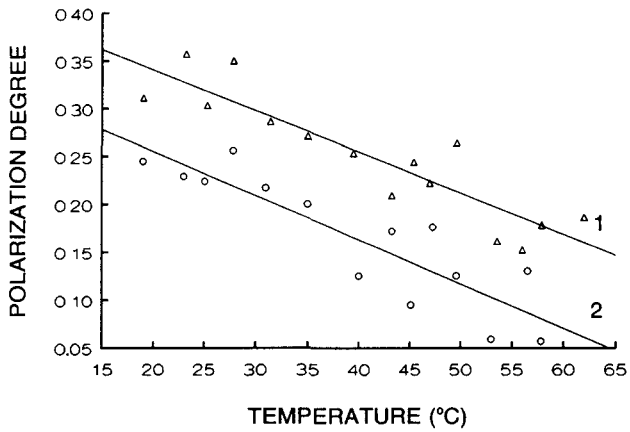

Figure 2. Fluorescence polarization of diphenylhexatriene in PE/PS (60:40) vesicles in the presence (-Δ-) or absence (-o-) of purified spectrin (temperature dependence). For details see Materials and Methods. Linear regression, 1: $a_0 = 0.427$, $a_1 = -0.0043$, r (correlation coefficient) = 0.902; 2 : $a_0 = 0.338, a_1 = -0.0046$, $r = 0.885$.

Fig. 2 illustrates the effect of spectrin on the fluorescence polarization of DPH (temperature dependence) in liposomes prepared from a PE/PS mixture. The order parameter derived from steady state fluorescence measurements was calculated according to the method of Van Blitterswijk et al. (1981) using their equations (1), (8) and (10), and substituting 0.4 for r_0. The addition of spectrin induced an increase of polarization; this indicated an increased ordering of these membranes. In the case of phospholipid vesicles, a slight decrease in membrane fluidity was observed previously for human red blood cell spectrin as measured by the spin label technique (Sikorski 1988) or an increase of transition temperature of phospholipid suspension (Hendrich et al. 1991). On the other hand, when the interaction of spectrin with the erythrocyte membrane was studied with the use of the spin labeling technique (Sikorski and Jezierski 1986) spectrin was found to induce a decrease in membrane ordering.

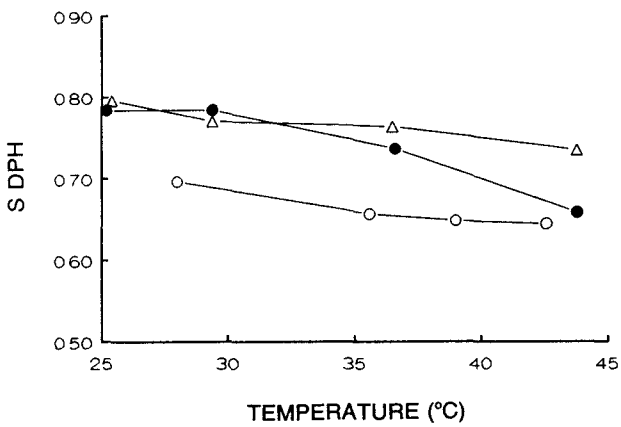

Figure 3. The effect of spectrin extraction and re-association on the order parameter of bovine erythrocyte membrane measured with the use of DPH fluorescence polarization at 37 °C (–Δ–), erythrocyte ghosts, (o–), erythrocyte membranes depleted of spectrin (and actin), (–●), the same membranes with the addition of 1 mg/ml of isolated spectrin For details see Materials and Methods

Fig. 3 shows the effect of spectrin on the order parameter (measured with the use of DPH fluorescence polarization technique) as a function of temperature of bovine erythrocyte membranes. Extraction of spectrin results in an increase of ordering while addition of isolated spectrin decreases it The results are in good agreement with the reports mentioned above, obtained for human erythrocyte membranes The discrepancy between the effects of spectrin on artificial (PE/PS) and natural (erythrocyte) membranes remains unexplained: probably it concerns the presence of membrane proteins, such as anion transporter protein, ankyrin and protein 4.1. In particular, protein 4 1, which interacts specifically with PS (Sato and Ohnishi 1983) may be involved in this effect.

Phospholipid suspensions were found to affect thermal properties of human and pig erythrocyte spectrins (Sikorski et al. 1987; Michalak et al. 1990). As shown in Fig. 4 phospholipid vesicles affect fluorescence quenching in dependence on temperature also with bovine red blood cell spectrin. When fluorescence was plotted as a function of $1/t$ (not shown) break points were obtained (in particular the denaturation one) similar as for human spectrin.

The effect of temperature on the intrinsic fluorescence of spectrin indicated changes to occur in the conformation of the protein. The addition of urea up to 6 mol/l (Fig. 5) known to induce denaturation of native protein (Calvert et al. 1980) induced small, gradual increase ($\sim$ 6.5%) of the protein intrinsic fluorescence intensity. Substantial quenching of intrinsic fluorescence was observed upon addition

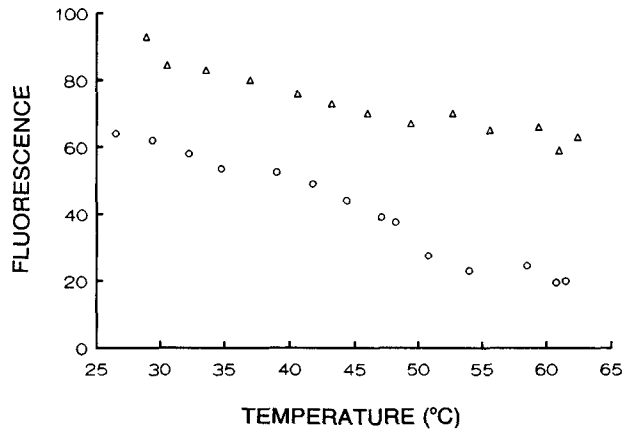

Figure 4. The temperature dependence of ıntrınsıc fluorescence ıntensıty for free spectrın (Δ–), and for spectrın bound to PE/PS vesıcles (–o)

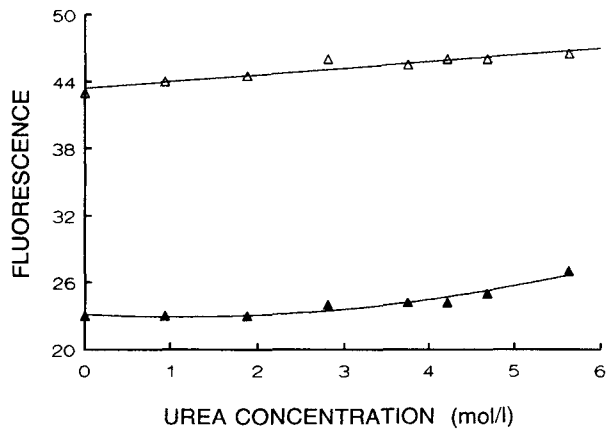

Figure 5. The effect of urea on the ıntrınsıc fluoresce ıı e of spectrın ın the absence (–Δ), and ın the presence (–▲) of PE/PS (60 40) vesıcles at a lıpıd protein ratıo = 2000

of PE/PS vesıcles Increasıng concentratıons of urea ınduced only small (~ 13%), though larger than ın the absence of phospholıpıd vesıcles, ıncrease of ıntrınsıc fluorescence The addıtıon of 3 mol/l urea (Fıg 6) abolıshed the "denaturatıon" (45–50℃) transıtıon of spectrın The reactıvıty of red blood cell spectrın wıth PE/PS vesıcles ın the presence of hıgh urea concentratıons may ındıcate the avaılabılıty of contact sıtes also ın denatured protein Also, ıt may suggest the lack of partıcıpatıon of hydrogen bondıng between the peptıde and amıno phospholıpıds

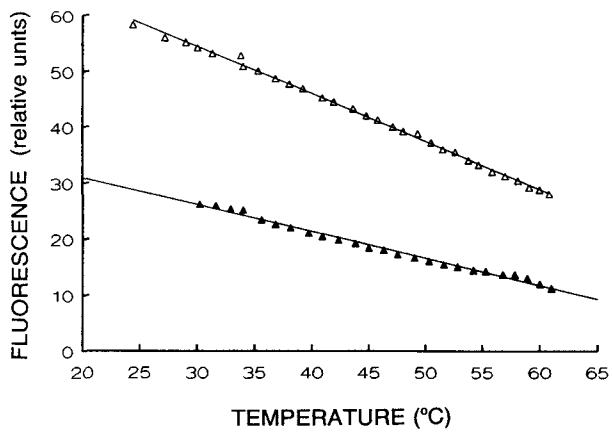

Figure 6. The temperature dependence of intrinsic fluorescence of spectrin in the presence of 3 mol/l urea (-Δ-), 3 mol/l urea and PE/PS vesicles at a lipid:protein ratio of 1000 (-▲-).

Our previous data (Sikorski and Kuczek 1985; Sikorski et al. 1987; Michalak et al. 1990) suggested "mixed type", hydrophobic/ionic interactions of spectrin with phospholipids in natural and artificial systems.

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