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Interaction of spectrin with phospholipids. Quenching of spectrin intrinsic fluorescence by phospholipid suspensions

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Phospholipid suspensions prepared of phosphatidylethanolamine, phosphatidylserine and their mixtures are able to influence the intrinsic protein fluorescence of spectrin. In the case of phosphatidylethanolamine suspension up to 75% of protein fluorescence can be quenched. The interaction of phospholipid aggregates with spectrin is modulated by pH and ionic strength. Phospholipids, particularly phosphatidylethanolamine display a 'stabilizing' effect against the changes of protein fluorescence induced by increasing ionic strength and by thermal denaturation.

Introduction

In mammalian erythrocytes, the membrane skeleton is a complex formed by peripheral membrane proteins anchored to the membrane intrinsic domain through the interactions with one or more integral membrane proteins. This complex is believed to be essential in maintaining cell shape, in the re-establishment of shape following deformation, and in influencing the distribution and mobility of membrane proteins and phospholipids (for a review see Refs. 1 and 2). Spectrin, which accounts for 75% of the mass of the cytoskeleton of the erythrocyte membrane, is composed of two nonidentical subunits, α and β, of M_r 240000 and 220000, respectively [3]. Spectrin forms heterodimers which are long, flexible rods of 100 nm contour length [4]. Heterodimers associating head-to-head form $(\alpha\beta)_2$ tetramers (200 nm long) which appear to be the physiologically relevant unit of spectrin in the erythrocyte membrane [5,6]. The skeletal complex is connected to the hydrophobic domain of the membrane lipids and intrinsic proteins by protein-protein and protein-lipid interactions. The main receptor of spectrin is ankyrin (band 2.1 protein) [7,8] which simultaneously binds to the anion transporter (band 3 protein). On the other hand there are indications from model studies [9–15] and from the research on the cells and/or isolated erythrocyte membranes that spectrin interacts with membrane lipids [16–19]. There are also data suggesting the influence of lipidic domain on the structure and shape of the membrane skeleton [20]. Moreover, studies on spectrin structure showed that part of its molecule is markedly hydrophobic [21], although its hydrophobicity index is lower than those of, for example, actin and serum albumin [22]. Isenberg et al. [22] showed that spectrin contains a large number of binding sites for hydrophobic ligands. In our experiments we found that phospholipid suspen-

Abbreviations used: PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine.

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sions particularly prepared of phosphatidylethanolamine (PE) and its mixtures with other phospholipids are able to influence the protein fluorescence of spectrin. This indicates a direct specific interaction between spectrin and these phospholipids. Moreover, the suspensions of phospholipids used in this study display a 'stabilizing' effect against the changes of protein fluorescence caused by increasing the ionic strength of the solution.

Materials and Methods

Erythocyte ghosts were isolated from recently out-dated blood obtained from the local blood bank, according to Dodge et al. [23]. Spectrin dimer was extracted from erythrocyte ghosts at 37 °C for 30 min with 0.1 mM EDTA in 0.3 mM phosphate buffer (pH 7.2) containing 50 μl/ml of phenylmethylsulfonyl fluoride. The concentrated extract was chromatographed on Sepharose CL-4B column (55 × 1.6 cm) equilibrated with 5 mM sodium phosphate buffer (pH 7.2) containing 50 mM NaCl/0.1 mM 2-mercaptoethanol/0.1 mM EDTA. For studies of spectrin-lipid interactions at low ionic strength, NaCl was omitted from the column buffer. The second peak consisting of spectrin dimer was collected, pooled and used for the fluorescence measurements. Purity of spectrin was tested in (0.1%) SDS (5.6%) - polyacrylamide gel electrophoresis [24]. For details see Ref. 19. Protein concentration was determined according to Mejbaum-Katzenellenbogen [25]. The pH of the solutions was adjusted with 0.5 M Tris-phosphate buffer containing 0.25 M NaCl, so that final concentration was as follows: 0.1 M Tris phosphate/0.05 M NaCl/0.1 mM EDTA/0.1 mM 2-mercaptoethanol. If the influence of ionic strength was to be studied the phosphate buffers of concentration and pH as indicated in the legends to Figs. 3 and 4 were used and the appropriate amount of NaCl was added. Purified spectrin dimer was used within 2 days after isolation. To the 400 μl spectrin sample in appropriate buffer up to a maximum of 25 μl of phospholipid suspension was added and the fluorescence measurements were performed after at least 15 min at room temperature. Fluorescence measurements were performed with a Perkin-Elmer MPF-3L spectrofluorimeter equipped with a temperature controlling device. The temperature of the sample was measured with a digital thermometer with a platinium resistance sensor. The excitation wavelength was 290 nm and the emission maximum was found to be 337 nm.

Phosphatidylserine (PS) and phosphatidylethanolamine (from bovine brain) were purchased from Koch-Light (Colnbrook, U.K.), egg yolk phosphatidylcholine (Fluka, A.G. Buchs Switzerland) additionally purified, was a kind gift of Dr. A. Kozubek, Institute of Biochemistry, University of Wrocław, Poland. All lipids were tested in thin-layer chromatography on silica gel plates (Kieselgel 60 Merck, Darmstadt, F.R.G.) in chloroform/methanol/water (65 : 25 : 4). Phospholipid suspensions were prepared by evaporating chloroform from an appropriate amount of phospholipid solution. The evaporation was carried out for 1–2 h with the use of a glass evaporator connected to the oil vacuum pump. Phospholipids were then shaken with 12.5 mM borate buffer (pH 8.0) for 40 min. The suspension was centrifuged at 12 500 × g for 20 min and the supernatant was used for the experiments. If sonicated vesicles were to be prepared, the suspension was sonicated 20 min with an MSE Ultrasonic Disintegrator (20 kHz, London, U.K.). Phospholipid phosphorus was determined according to Bartlett [26].

Results and Discussion

If Sepharose CL 4B column-purified spectrin dimer is mixed with phospholipid suspensions prepared from PE, PS, phosphatidylcholine (PC) and of the mixture of PE with PS or PC (molar ratio 60 : 40), a change in intrinsic spectrin fluorescence is observed (Fig. 1). The greatest effect is caused by the suspension of PE which is able to quench as much as 48% of spectrin fluorescence at pH 7.7 and over 75% at pH 5.2 (Fig. 2). The effect of the PS suspension is not clear, since shaken suspension become turbid at higher concentrations, so that the increase in the fluorescence intensity may be an artifact. Sonicated PS vesicles, however, induce a rather slight decrease in the relative fluorescence intensity. The effect of PS suspensions is larger at lower pH (Fig. 2). The effect of large multilamellar vesicles prepared from egg yolk

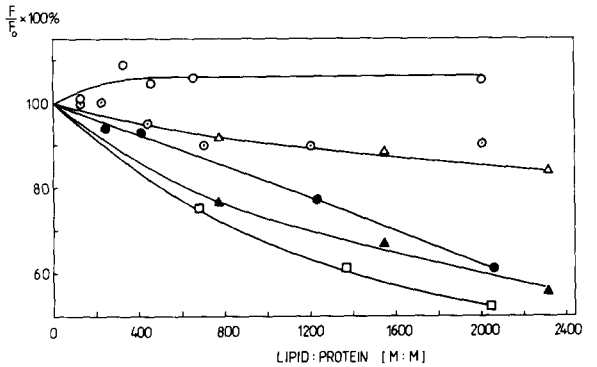

Fig. 1. Influence of suspensions of some phospholipids on the intrinsic fluorescence of spectrin. Spectrin samples were made up to 0.1 M Tris-phosphate buffer (pH 7.7) containing 0.05 M NaCl/0.1 mM EDTA/0.1 mM 2-mercaptoethanol. Excitation wavelength was 290 nm; emission wavelength (max) was 337 nm. ○, PS; ⊙, sonicated PS; ●, PE/PS mixture (60:40); spectrin concentration was $2.7 \cdot 10^{-7}$ M; △, PC; ▲, PE/PC mixture (60:40); spectrin concentration $2 \cdot 10^{-7}$ M; □, PE; spectrin concentration was $2.2 \cdot 10^{-7}$ M. Up to 25 μl of phospholipid suspension prepared as described in the text were added to 0.4 ml of the spectrin sample. Samples were incubated at room temperature for at least 15 min before measurements were performed. F and F_0, relative fluorescence intensities in the presence and in the absence of phospholipid suspension, respectively.

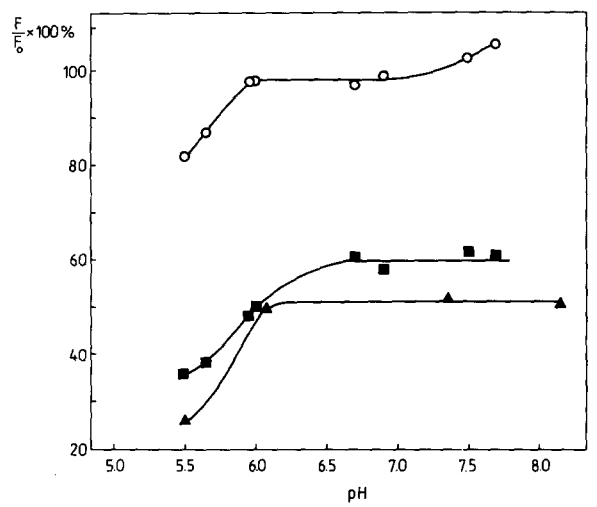

Fig. 2. pH dependence of spectrin fluorescence upon interaction with PS, PE and PE/PS (60:40) mixture suspensions. ○, spectrin with PS suspension (molar ratio 650); ▲, spectrin with PE suspension (molar ratio 2050); ■, spectrin with PE/PS mixture suspension (molar ratio 2050). Other details are as in Fig. 1.

phosphatidylcholine is rather low (about 15%) at a lipid/protein ratio of about 2000 at pH 7.7 and it is almost the same at lower pH (6.5, not shown). At higher ionic strength (about 0.4 M NaCl) phosphatidylcholine vesicles have no significant influence on spectrin fluorescence intensity (not shown). The mixtures of PE either with PC or PS have a rather great effect on the intensity of spectrin fluorescence. It should be noted that sonicated PS/PE vesicles showed almost the same effect on spectrin fluorescence intensity as the hand-shaken suspension (not shown).

One possible explanation of the observed quenching of protein fluorescence might be conformational change(s) in certain segment(s) of spectrin induced by binding of phospholipid bilayers. The emission maximum of spectrin did not shift upon the addition of phospholipid suspension in contrast to the other proteins interacting with the bilayer [27,28]. This might be due to large number of tryptophanyl residues (70) [22] present in the spectrin molecule. Different tryptophanyl residues might undergo different changes of disposition that result in the absence of the red shift of the emission maximum.

In Fig. 2 the results of the experiments on the influence of some phospholipid PE, PS and PS/PE mixture suspensions on the tryptophanyl fluorescence of spectrin as a function of pH are summarized. Similar curves were obtained at the other protein/lipid ratios (not shown). In all three cases analysed, the interaction expressed as the decrease in fluorescence intensity is proportional to the decrease of electrostatic repulsion between this acidic protein (isoelectric point 5.0–5.2 [29]) and phospholipids. Mombers et al. [13] found a sharp optimum at pH 5.5 for anionic phospholipid interaction with spectrin as measured by the spectrin penetration rate of lipid monolayers. Isenberg et al. [22] showed that brominated fatty acids are efficient quenchers of tryptophan fluorescence of spectrin. They found that binding of these ligands was predominantly hydrophobic which suggested the presence in the spectrin molecule of multiple hydrophobic sites. In our experiments very similar results are obtained with phospholipid suspensions, particularly PE and its mixtures with other phospholipids.

If palmitic acid was added (ethanolic solution),

a slight increase in fluorescence intensity at 32°C and about 10% quenching at 18°C at protein/ligand molar ratio 300–1300 in comparison to the control sample were observed. Linolenic acid, however, caused a significant (up to 26.7% at ligand to protein molar ratio of 1700) quenching of the tryptophan fluorescence of spectrin (data not shown). In Fig. 3a and b the spectrin intrinsic fluorescence quenched by the suspension of PS/PE mixture (60 : 40) at two different pH values in different salt concentrations is shown. The influence of the increasing salt concentration is larger

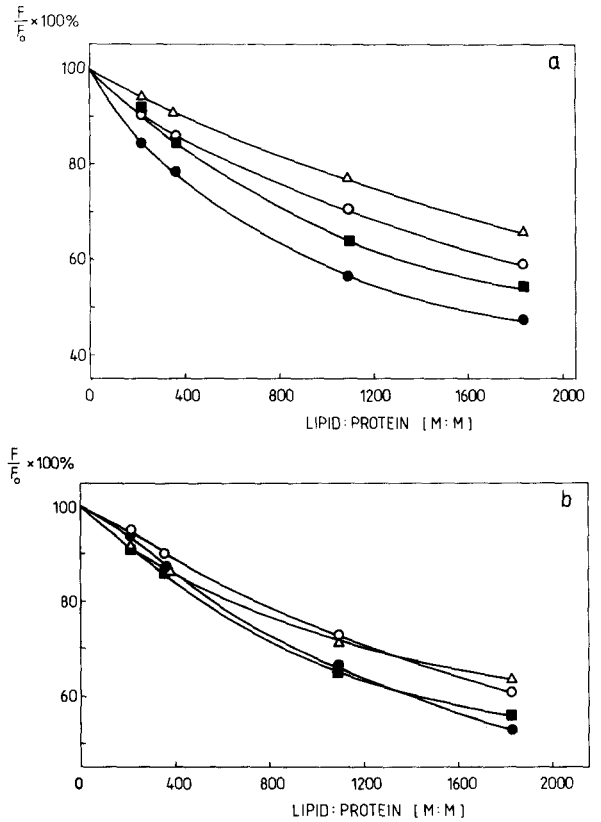

Fig. 3. Quenching of spectrin fluorescence by PE/PS (60 : 40) suspensions in solutions of different NaCl concentrations at (a) pH 6.25, 0.04 M phosphate buffer containing 0.1 mM EDTA, 0.1 mM 2-mercaptoethanol and indicated concentrations of NaCl. Protein concentration, $3 \cdot 10^{-7}$ M. (b) pH 7.5, 0.005 M phosphate buffer containing 0.1 mM EDTA, 0.1 mM 2-mercaptoethanol and indicated concentrations of NaCl. ●, 0 M NaCl; ■, 0.3 M NaCl; ○, 0.6 M NaCl; △, 1.0 M NaCl. Each experimental point was obtained by comparison of spectrin fluorescence at specified pH and NaCl concentrations in the absence (F_0) and in the presence (F) of PE/PS (60 : 40) vesicles.

at pH 6.25 (from over 50% to less than 30% quenching) than at pH 7.5 (from less than 50% to over 30 % quenching). It should be noted that in this experiment the fluorescence of purified spectrin is compared with the fluorescence of the same spectrin preparation in the presence of the phospholipid suspension at the same ionic strength. The influence of pH and, particularly, ionic strength on the interaction of phospholipid with spectrin might suggest a 'mixed' type of hydrophilic-hydrophobic interaction. Similar effects were also observed by others. Isenberg et al. [22] pointed out that at low ionic strength an expansion of the spectrin molecule takes place [30] which could result in the increase in the number of binding sites.

Intrinsic spectrin fluorescence decreases with the increasing ionic strength (up to 1.0 M NaCl). As is shown in Fig. 4, the fluorescence of the same spectrin sample (F) in 1.0 M NaCl is more than 30% lower than the initial fluorescence (F_0) of this protein in low ionic strength buffer. This event might be due to the conformational changes which are reported by Ralston and Dunbar [30] to take place with the changes of the ionic strength of the spectrin solution. If a phospholipid suspension

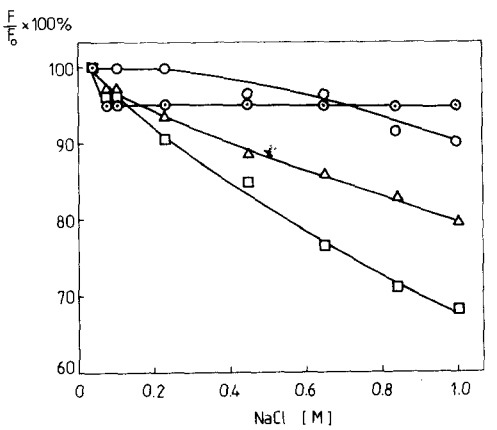

Fig. 4. Effect of NaCl on the intrinsic fluorescence of spectrin in the presence or absence of phospholipid suspensions. F_0, initial fluorescence of spectrin in 0.004 M phosphate buffer (pH 6.25) containing 0.1 mM EDTA/0.1 mM 2-mercaptoethanol. F, fluorescence measured at specified NaCl concentrations in the above buffer. □, spectrin; △, spectrin with PS suspension (molar ratio 400); ○, spectrin with PE/PS mixture suspension (molar ratio 1100); ⊙, spectrin with PE suspension (molar ratio 1250). Spectrin concentration, $3 \cdot 10^{-7}$ M.

(spectrin/phospholipid molar ratio about 1000) is present, the decrease in spectrin fluorescence is much smaller and in the case of PE and the mixture of PE/PS this decrease is in the range of 0–10% (Fig. 4). A similar but lower effect was observed at pH 7.5; e.g., F/F_0 for spectrin at 1.0 M NaCl was 0.66, while for spectrin with PE at a lipid/protein molar ratio of 1250 this value was about 0.82 (not shown). The 'stabilizing' effect of phospholipid suspensions on the spectrin molecule could be confirmed by the observation of the influence of temperature on the intrinsic fluorescence of spectrin in the presence of multilamellar liposomes prepared from a PE/PS mixture (Fig. 5). Isolated spectrin in conditions applied (0.15 M NaCl, pH 6.6) displays two 'breaks' – at about 29 °C and about 45 °C, which represents the denaturation of the protein [31]. This is about 3–4 Cdeg lower than observed by other methods. Perhaps the reason is that the experiment was performed at pH 6.6. The 29 °C 'transition' might represent a conformation change preceding association of dimers to tetramers [32]. At higher temperatures spectrin solutions become turbid. If phospholipid vesicles (PE/PS, 60:40) are added at a lipid/protein molar ratio of about 2000 the 'breaks' shift to lower temperatures and above the second (denaturation) 'break' the fluorescence decreases. The spectrin solution is still transparent

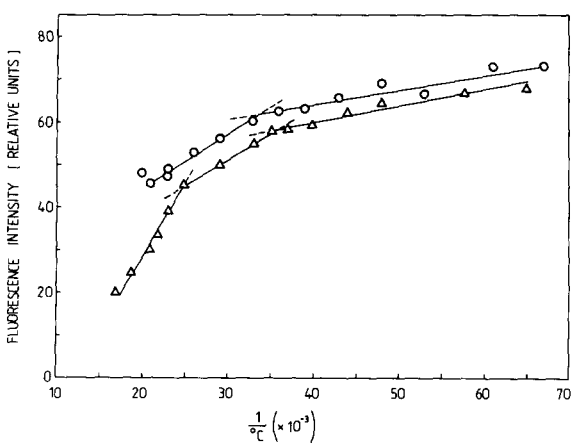

Fig. 5. Temperature dependence of the relative fluorescence intensity of spectrin [$F = f(1/t)$] in the absence (○) and in the presence (△) of PE/PS (60:40) mixture suspension. Molar ratio spectrin/phospholipid 2000; 0.04 M phosphate buffer (pH 6.6) containing 0.15 M NaCl/0.1 mM EDTA/0.1 mM 2-mercaptoethanol; spectrin concentration, $1 \cdot 10^{-7}$ M.

and precipitation of spectrin is not observed even at temperatures higher than 70 °C.

In several model studies spectrin was shown to penetrate phospholipid monolayers and to bind to multilammelar and unilamellar liposomes thereby inducing the changes in their permeability and thermotropic properties [9–11,12–15].

In the present study we found that phospholipid suspensions are able to quench the intrinsic protein fluorescence of spectrin that is supposed to be a result of binding of phospholipid aggregates to spectrin. In model studies and in the studies on cells the specificity of interaction of spectrin with PS [9,16,17] and also with its mixtures with PE [14] mimicking the composition of the inner layer of the erythrocyte membrane were observed. In this study, we also paid special attention to the interaction of vesicles prepared from these phospholipids. However, unlike in the above mentioned studies, the greatest effects are observed when PE suspensions interacted with spectrin. Also, the efects are easily observable at physiological pH and ionic strength. The reason for these discrepancies has not yet been established. The differences in the length and unsaturation of acyl chains of phospholipids used by cited authors and in this study might at least in part explain the above-mentioned differences. The role of unsaturation of acyl chains could be confirmed by the effects given above of fatty acids on spectrin's intrinsic fluorescence.

The biological significance of the 'stabilizing' effect of phospholipids on spectrin has not yet been explored. Further detailed, conformational studies should be performed for its elucidation.

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