Investigation of Spectrin Binding to Phospholipid Vesicles Using Isoindole Fluorescent Probe. Thermal Properties of the Bound and Unbound Protein

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Abstract. Fluorescence of isoindole probe covalently bound to spectrin from pig erythrocytes, and fluorescence of tryptophanyl residues were used to study spectrin interaction with phospholipid bilayers. Evidence would be provided for conformational changes of spectrin occurring upon its binding to lipid bilayers. Fluorescence quenching experiments allowed to determine thermal stability of the protein in bound and unbound state. Spectrin binding to lipids was shown to protect the protein against thermal denaturation.

Key words: Spectrin — Conformational changes — Phospholipid bilayers — Fluorescence probe — Fluorescence quenching

Introduction

Spectrin is the major structural protein of erythrocyte membranes. It is composed of two subunits, α and β, with molecular weights of 240 kDa and 220 kDa respectively (Clark 1971). Both subunits form spectrin heterodimers which are long, flexible rods of approx. 100 nm lenght (Shotton et al. 1979). In the cell, spectrin is present almost entirely in the form of tetramer, containing two heterodimers (Ji et al. 1980).

Spectrin has been assumed to play essential role in maintaining the shape of erythrocytes allowing it the property of reversible deformability (Steck 1974). Spectrin binds to the erythrocyte membrane mainly via its interactions with membrane proteins (Bonnet and Begard 1984; Mombers et al. 1979). However, studies of interactions between spectrin and model lipid membranes have revealed association of the protein with lipids (Mombers et al. 1979, 1980). In a preceding paper (Sikorski et al. 1987) the effects of factors such as pH, ionic strength and phospholipid type used to form bilayers from, on spectrin associa-

tion with membranes, were reported. Lipids-induced quenching of tryptophanyl residues under spectrin binding to phospholipid vesicles was used to study the interactions. Pronounced effects were observed in particular for phosphatidylethanolamine (PE) and a mixture of phosphatidylethanolamine and phosphatidylserine (PE/PS).

The aim of the present study was to show whether spectrin interaction with lipid bilayers may induce changes in the protein conformation. In our experiments spectrin was labelled with isoindole fluorescent probe covalently bound to the protein molecule. This fluorescence probe was successfully used for studying conformational changes of other proteins, e.g. glyceraldehyde-3-phosphate dehydrogenase from rabbit muscle (Michalak et al. 1987). The interactions with membranes and conformational changes of proteins can be recorded by monitoring both the fluorescence of the labelled protein and the resonance energy transfer between tryptophanyl residues and the fluorescent probe. Additionally, the method of evaluation of fluorophores exposition described by Eftink and Ghiron (1976, 1977) was employed in the present study. Non-ionic quencher, acrylamide, was used as the quencher of tryptophanyl and isoindole fluorescence.

The effect of temperature on spectrin structure has been reported by several investigators who used circular dichroism, scanning calorimetry (Brandts et al. 1977) and fluorescence polarization (Yoshino and Marchesi 1984) to study changes in the protein structure. In the present work, the effect of spectrin binding to phospholipid bilayers on thermal stability of the protein was shown. Changes of spectrin conformation upon heating in bound and unbound state were examined by acrylamide-induced fluorescence quenching of tryptophanyl residues and the isoindole probe.

Materials and Methods

Materials: Erythrocyte ghosts were isolated from fresh pig blood according to Dodge et al. (1963). Spectrin dimers were extracted from erythrocyte ghosts at 37 °C for 30 min with 0.3 mmol/l phosphate buffer (pH 7.2), containing 0.1 mmol/l EDTA and 13 μmol/l phenylmethylsulfonyl fluoride. The concentrated extract was chromatographed on a Sepharose CL-4B column (55 × 1.6 cm) equilibrated with 12.5 mmol/l borate buffer (pH 8.0), containing 0.14 mol/l NaCl, 0.1 mmol/l 2-mercaptoethanol, 0.1 mmol/l EDTA. The purity of spectrin was tested by 0.1 % SDS (5.6 %) — polyacrylamide gel electrophoresis (Fairbank et al. 1971). Only two bands α and β were obtained with no trace of any other proteins. The protein concentration was determined according to Mejbaum—Katzenellenbogen (1955). Purified spectrin dimers were used within two days of isolation. In all experiments the protein concentrations ranged between 1.3—1.5 × 10^{-7} mol/l, and the lipid to protein molar ratio was 700.

Phosphalidylserine (PS) and phosphatidylethanolamine (PE) from bovine brain were purchased from Koch-Light Laboratories. The purity of lipids was checked by thin-layer chromatography

on silica gel plates (Kiesel-gel 60 Merck) in a chloroform/methanol/water mixture. Acrylamide was purchased from Bruxelles r.b.c. and was recrystallized before use. Other chemicals were of analytical grade.

Vesicle preparation: Phosphatidylserine solution was evaporated for 1—2hours in a glass evaporator connected with an oil pump. Phosphatidylethanolamine was added to evaporated phosphatidylserine (weight ratio PE:PS = 60:40) and then phospholipids were shaken with 12.5 mmol/l borate buffer (pH 8.0) for 40 min. The suspension was centrifuged at 12.500 × *g* for 20 min to remove large lipid aggregates. The phospholipid concentrations were calculated from phosphorus determination according to Bartlett (1959).

Spectrin labelling: Spectrin was labelled with *o*-phthaldialdehyde by the method used for trypsin and glyceraldehyde-3-phosphate dehydrogenase as described elsewhere (Sidorowicz and Michalak 1985; Michalak et al. 1987). In the present study the molar ratio of the fluorescence probe to protein was usually 30. Spectrin labelling with the isoindole probe did not affect the position of the emission maximum of the native protein fluorescence.

Fluorescence measurements: Fluorescence was measured with a Perkin—Elmer MPF-3L spectrofluorimeter. The fluorescence of protein tryptophans and the isoindole probe was excited at 290 and 340 nm, respectively. The widths of the excitation and emission slits was set to 7 nm. The spectrofluorimeter was equipped with a temperature controlling device. The sample temperature was measured directly with a platinium resistance sensor immersed into the cuvette containing the solution. Fluorescence polarization was measured with a polarization device in cuvettes of 0.4 ml volume.

Results and Discussion

The reaction of *o*-phthaldialdehyde with aminogroups of protein yields a highly fluorescent product (Beeler and Churchich 1978). It has the structure of isoindole and is covalently bound to protein. Isoindole was used to label spectrin molecules; this resulted in the appearance of a new band in the absorption spectrum of spectrin at 337 nm. A strong fluorescence band with an emission maximum at 437 nm was observed when the protein solution was excited at 340 nm. The intensity of the new band depended on the degree of the protein labelling.

An effect of spectrin association with PE/PS phospholipid vesicles on the polarization degree of the isoindole probe was observed. Addition of lipid vesicles to labelled protein solution increased the degree of polarization (from 0.14 to 0.29) (Fig. 1). It is well known (Shinitzky and Barenholz 1978) that the degree of polarization of a fluorophore changes in dependence on changes in the chromophore microenvironment, especially if microviscosity changes. The observed increase of the polarization degree of the isoindole probe in spectrin bound to vesicles may be due to conformational changes of the protein upon its

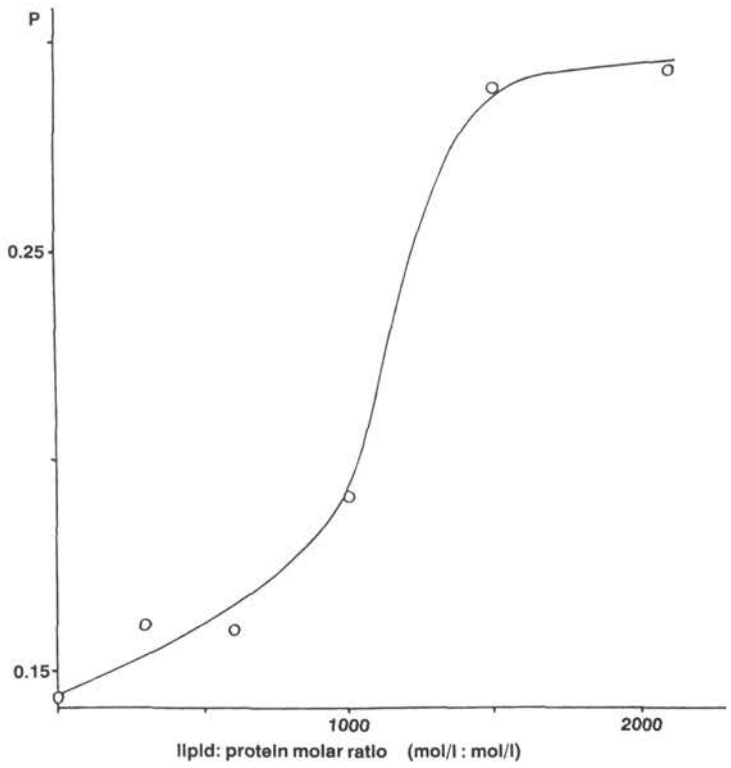

Fig. 1. Polarization degree (p) of isoindole probe in labelled spectrin as a function of lipid: protein molar ratio. Excitation wavelenght $\lambda_{ex} = 340$ nm.

association with lipids, or it may result from the contact of the fluorophore with the lipid bilayer.

To address the question whether spectrin changes its conformational state upon binding with membranes, the acrylamide-induced quenching of tryptophanyl residues and isoindole probe fluorescence was compared for free spectrin in solution and for spectrin bound to lipid bilayers.

Fig. 2 shows Stern—Volmer plots of acrylamide-induced fluorescence quenching for both fluorophore types in protein at room temperature.

In the presence of phospholipid vesicles, the quenching of the isoindole probe fluorescence was enhanced. The effect was much weaker with the tryptophanyl residues fluorescence. However, the enhanced quenching of the protein tryptophans fluorescence was observed in the presence of liposomes at all temperatures ranging between 15—60 °C (Fig. 4). The acrylamide-induced

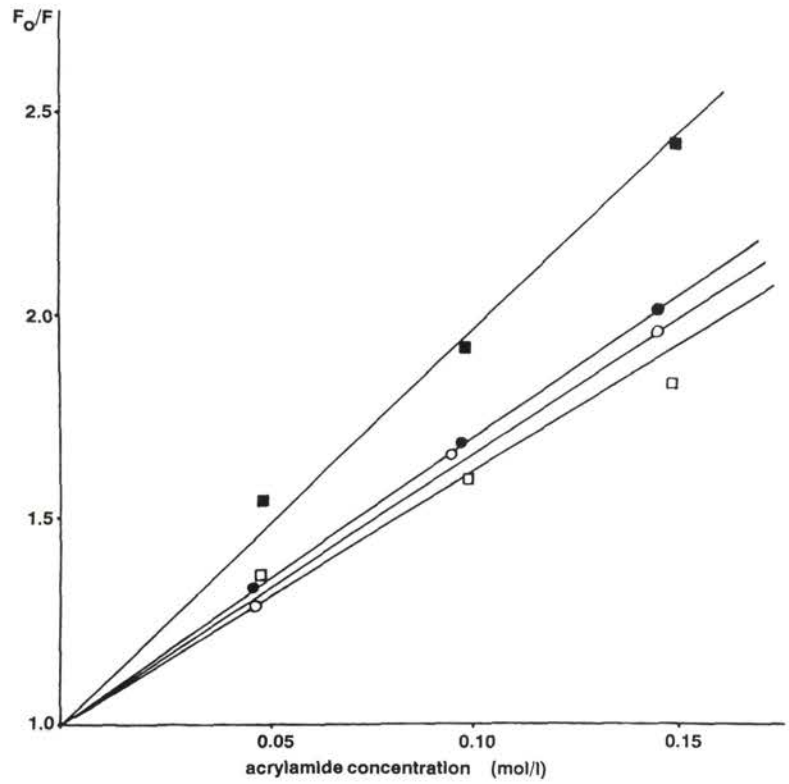

Fig. 2. Stern-Volmer plots of acrylamide-induced fluorescence quenching of tryptophanyl residues and isoindole probe for unbound spectrin and spectrin associated with PE/PS lipid vesicles. F_o, F — fluorescence intensities in the absence and in the presence of the quencher. (□) quenching of probe fluorescence in unbound spectrin and (■) in spectrin bound to PE/PS vesicles, $\lambda_{ex} = 340$ nm; (○), (●) quenching of tryptophan fluorescence in unbound and bound spectrin, respectively, $\lambda_{ex} = 290$ nm.

quenching enhancement of the fluorescence of both kinds of fluorophores in spectrin molecules suggests conformational changes of the protein occurring upon its binding to lipid membranes. A trivial screening effect of the lipid bilayer environment should result in less accessibility of acrylamide from water solution to protein chromophores.

To obtain additional information about the changes in spectrin conformation induced by its binding to membranes, the effects of PE/PS vesicles and urea on the labelled protein fluorescence were compared. Urea at 2 to 4 mol/l induces unfolding of spectrin molecules (Yoshino and Marchesi 1984). Excitation of the

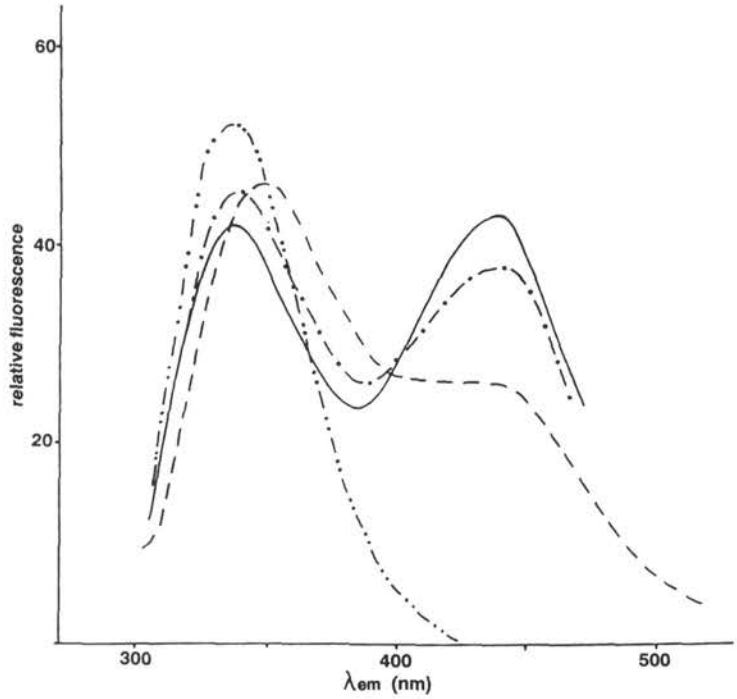

Fig. 3. Effect of urea on labelled spectrin fluorescence spectrum. (— · · —) nonlabelled spectrin, (——) spectrin labelled with *o*-phthaldialdehyde, (— · —) labelled spectrin after addition of 2 mol/l urea, and (— — —) 4 mol/l urea, $\lambda_{ex} = 290$ nm.

labelled protein at 290 nm results in a resonance energy transfer between tryptophanyl residues and the isoindole probe. The fluorescence of tryptophanyl residues is weaker in comparison with that of the unlabelled protein, and a strong fluorescence of the isoindole probe appears with an emission maximum at 437 nm (Fig. 3). The occurrence of resonance energy transfer was confirmed by the appearance of an additional band in the excitation spectrum of the isoindole probe (acceptor) in the range of donor absorption (295 nm). In a previous work (Michalak et al. 1987) we could demonstrate that the efficiency of the energy transfer between tryptophanyl residues and insoindole fluorophores in glyceraldehyde-3-phosphate dehydrogenase may be a good indicator of conformational changes of the enzyme. Fig. 3 illustrates the effect of urea on the fluorescence spectrum of labelled spectrin. The decreased intensity of isoindole fluorescence at 437 nm with a simultaneous increase of that of tryptophan is connected with a change of the energy transfer efficiency between the fluorophores under the action of the denaturating agent.

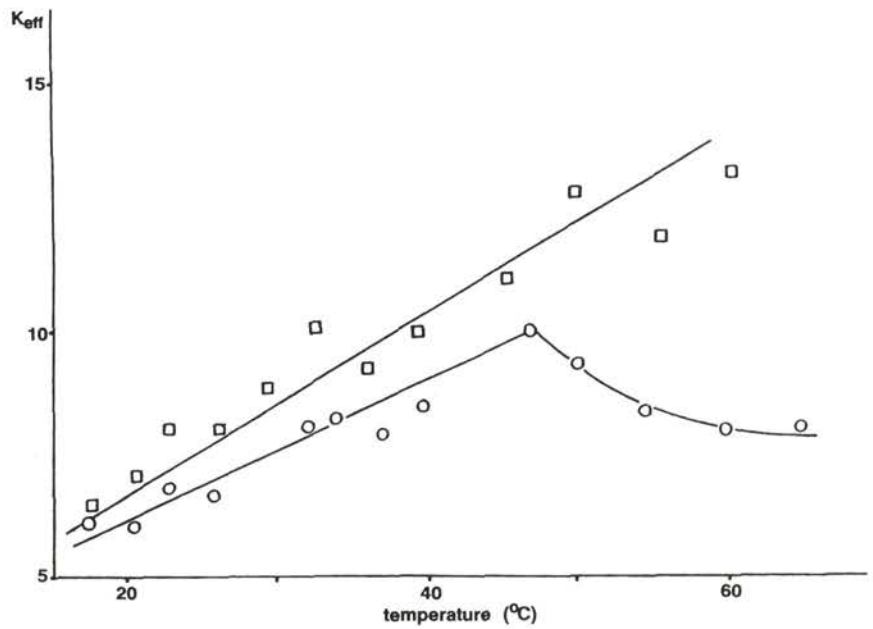

Fig. 4. Effective quenching constant (K_{eff}) of acrylamide-induced fluorescence quenching of tryptophanyl residues in spectrin as a function of temperature. (○) non-associated spectrin. (□) spectrin associated with phospholipid vesicles, $\lambda_{ex} = 290$ nm.

Upon the association of spectrin with lipid membranes, the intensity of isoindole fluorescence (excited at 290 nm) was the same as for free labelled spectrin. The efficiency of energy transfer did not vary. It may be suggested that the changes in the protein conformation are not as marked as those induced by urea.

Also, the effect of spectrin binding to PE/PS vesicles on the thermal stability of the protein was examined. Acrylamide-induced quenching of tryptophan and isoindole fluorescence of spectrin was used as a measure of the accessibility of both fluorophore types for the quencher. This method was described by Eftink and Ghiron (1976) for multitryptophan proteins. For some multitryptophan proteins plots of F_0/F as a function of quencher concentration $[Q]$ is not linear (F_0 and F have the same meaning as described in legend to Fig. 2). Information about the fluorophore exposition may be obtained from the initial slope of the Stern—Volmer plots. From the initial slopes, effective quenching constants (K_{eff})

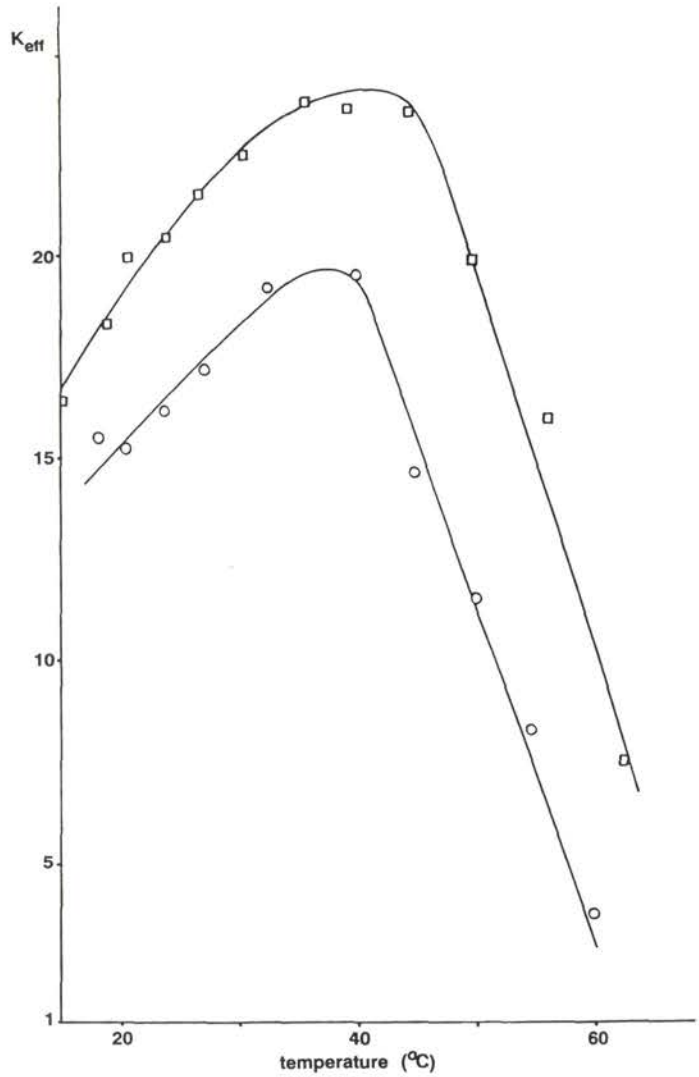

Fig. 5. Effective quenching constant (K_{eff}) of acrylamide-induced fluorescence quenching of isoindole probe as a function of temperature. (○) non-associated spectrin, (□) spectrin associated with phospholipid vesicles. $\lambda_{ex} = 340$ nm.

were calculated. Stern—Volmer plots obtained from our calculations were linear at low acrylamide concentration for both fluorophore types in spectrin molecules (Fig. 2). At higher acrylamide concentrations the curves bent downwards. Effective quenching constants K_{eff} were calculated from initial slopes of

plots for tryptophanyl residues and isoindole probe in the range of temperatures between 15 °C and 60 °C.

Upon heating, a monotonic increase of accessibility for the quencher initially was observed for both tryptophanyl residues and the isoindole probe in the unbound state of spectrin. Above a definite temperature in the range of 40 °C to 47 °C, a rapid decrease in accessibility occurred (Fig. 4, 5). In the presence of lipid vesicles, this transition was shifted toward higher temperatures. This suggests that the thermal stability of spectrin increases upon its association with lipid bilayers.

Yoshino and Minari (1987) observed dissociation of spectrin dimers from human erythrocytes to monomers at 49 °C. Also, other authors (Knowles 1983) described a sharp structural transition at 49 °C, suggesting the occurrence of nonreversible changes in the secondary structure of spectrin molecules.

In our experiments, conformational changes of unbound spectrin were observed at lower temperatures. This difference may be due to differences in spectrins from pig and human erythrocytes, or may be connected with the high sensitivity of the fluorescence method. Similarly, Yoshino and Marchesi (1984) observed a significant decrease of polarization anisotropy of tryptophan fluorescence of spectrin from human erythrocytes in the range of 38 °C—48 °C. Circular dichroism and scannig calorimetry studies (Brandts et al. 1977; Calvert et al. 1980) indicate that upon heating in these range of temperatures, a gradual unfolding of the spectrin molecule occurs.

In conclusion, the observation of fluorescence of the isoindole probe and of tryptophanyl residues in spectrin may be useful for monitoring of the protein interactions with lipid bilayers and may help in detecting conformational changes of the protein upon its association with them. Conformational changes induced in spectrin molecules upon its binding to membrane are less marked than those induced by urea. Moreover, acrylamide-induced fluorescence quenching experiments allow to study the thermal stability of bound and unbound protein. Phospholipid suspensions have a "stabilizing effect" on spectrin molecules upon its heating up to about 60 °C.

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