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INTERACTION OF ANILINONAPHTYL LABELED SPECTRIN WITH FATTY ACIDS AND PHOSPHOLIPIDS : A FLUORESCENCE STUDY

Didier BONNET [*+] and Evelyne BEGARD [*]

[+] Museum National d'Histoire Naturelle,
Chaire de Physicochimie de l'adaptation, 75005 PARIS FRANCE

[*] Institut de Biologie Physico-Chimique, Unité 128 INSERM,
13 rue Pierre et Marie Curie, 75005 PARIS FRANCE

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Summary. Anilinonaphtyl labeled spectrin exhibits a fluorescence emission spectrum characteristic of a highly hydrophobic environment. Quenching of the fluorescence intensity by nitroxide analogs of fatty acids of affinity 10^4 M^{-1} reveals that the sites of interaction of fatty acids lie very close to the anilinonaphtyl groups. Similar experiments performed with a nitroxide analog of phosphatidylserine yield a 30 % quenching of fluorescence while the same phosphatidylcholine analog has essentially no effect. The changes in the fluorescence emission spectrum exhibited in the presence of sonicated phosphatidylserine vesicles further outline the specificity of interaction towards phosphatidylserine, with one spectrin binding site per about 750 exposed phospholipids. Moreover, they suggest a penetration of the anilinonaphtyl group into the lipid bilayer

The erythrocyte membrane skeleton, a proteic network underlying the plasma membrane is involved in a number of physiologically-relevant properties (1) : restriction of integral membrane protein mobility, regulation of erythrocyte shape and deformability and maintenance of the transbilayer phospholipid asymmetry. How these functions are performed remains to be elucidated, but they seem to be based on non-covalent interactions between the skeleton and the membrane. Much work is currently being done to characterize them.

Spectrin, the major protein (by weight) of the membrane skeleton is an heterodimer of 460000 M.W. It is bound to the membrane at the anion-transport protein (band 3) by the intermediacy of a protein named ankyrin (2). Bands 4.1, two phosphoproteins of the skeleton, could constitute another link between spectrin and the membrane (3). Besides these interactions, spectrin was shown by distinct approaches including fluorescence quenching by brominated fatty acids (4) and penetration into phospholipid monolayers (5) to possess binding sites for negatively charged lipids. These

Abbreviations: EDTA, ethylene diamine tetraacetic acid, disodium salt; (0,2) P.C., 1-palmitoyl-2-(4-doxylpentanoyl) phosphatidylcholine; (0,2) P.S., 1-palmitoyl-2-(4-doxylpentanoyl) phosphatidylserine; (10,3) F.A., 5-doxylpalmitoic acid; (1,14) F.A., 16-doxylstearic acid; (1,14) P.C., 1-palmitoyl-2-(16-doxylstearoyl)-phosphatidylcholine.

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studies suggest that spectrin-lipid interactions could play some role in the binding of the membrane skeleton to the erythrocyte membrane.

In this work, spectrin is covalently labeled with the fluorescent anilinonaphtyl probe. Interactions between spectrin and lipids both in solution or in bilayers are studied by detecting changes in the fluorescence emission spectrum of the anilinonaphtyl group and quenching by spin labeled lipids (6). This direct approach yields affinity constants and moreover indicates a penetration by spectrin within the lipid bilayer.

MATERIALS AND METHODS

Spectrin preparation. Spectrin tetramers were prepared essentially as described (7) with the two following modifications. First, all buffers were made 0.5 mM in dithiothreitol except the extraction buffer (0.3 mM Na_2HPO_4/ NaH_2PO_4 pH = 7.6) which was 0.05 mM. Secondly, spectrin was concentrated by ultrafiltration in an Amicon cell (PM 30 Amicon membrane). Its purity was checked by slab gel electrophoresis using the buffer system of Laemmli (8).

Spectrin labeling. After an overnight dialysis against one liter of dialysis buffer : Tris, HCl 25 mM, EDTA 5 mM, NaCl 100 mM (pH = 7.5), 1 ml of 4×10^{-6} M spectrin was labeled by incubation at 30 °C for about 4 hours in the presence of 3×10^{-5} M N-(4 Anilino-1 Naphtyl) maleimide (Fluka) added as a 2.8×10^{-3} M acetonic solution. Since maleimides are not fluorescent, the labeling reaction was followed by the progressive increase of the fluorescence emission intensity. Incubation was stopped when no further increase was observed. Spectrin was finally dialyzed twice against 500 ml dialysis buffer.

Lipids. Phosphatidylcholine was purified from egg yolk according to Singleton et al. (9). Phosphatidylserine was prepared from phosphatidylcholine by transphosphatidylation catalyzed by phospholipase D and purified on CM-52 cellulose (Whatman) (10). Nitroxide analogs of phospholipids : (0,2) P.S. ; (0,2) P.C. ; (1, 14) P.C. and fatty acids (10,3) F.A. and (1, 14) F.A. were synthetized as described elsewhere (11, 12).

Vesicles were prepared by sonication at 0 °C under an Argon atmosphere of a phospholipid dispersion in dialysis buffer. Sonication was performed during 5 min at 40 V with an Ultrasons-Annemasse apparatus using a 3 mm diameter probe.

Fluorescence experiments. Before fluorescence measurements, spectrin solution at the required concentrations were filtrated on disposable filterholders (0.45 µm Schleicher-Schüll). Fluorescence experiments were performed on an S.L.M. 4800. The lamp emission spectrum was corrected by recording the ratio of the fluorescence intensity of the sample to a Rhodamin-B standard. Excitation and emission slits were respectively 4 and 2 nm in all experiments Emission intensity spectra were recorded at an excitation wavelength of 360 nm. The sample cell was jacketed and thermostated at 30 °C.

RESULTS

Characteristics of anilinonapthyl labeled spectrin

The number of anilinonaphtyl groups covalently bound to spectrin was evaluated by measuring the absorption of labeled spectrin at 350 nm assuming an $\varepsilon = 13180$ (12) : it was found to be equal to eight bound

residues per spectrin dimer. The two subunits were shown to be labeled by U.V. irradiation of an S.D.S. electrophoresis gel (5 - 10 % acrylamide gradient).

Anilinonaphtyl derivatives possess fluorescence emission spectra that are very sensitive to the polarity of their neighbourhood (13) : the quantum yield increases with the hydrophobicity (up to 10^2 times) while the maximum emission wavelength exhibits a pronounced blueshift (up to 80 nm).

The fluorescence excitation and emission maxima of anilinonaphtyl spectrin are respectively λ_{EX} = 370 nm and λ_{EM} = 438 nm. The emission maximum is characteristic of an environment with an hydrophobicity close to 2 methyl 2 propanol. This high hydrophobicity prompted us to look for a possible interaction of lipids with spectrin at the labeled area.

Binding of spin labeled fatty acids

Two fatty acid analogs (10,3) F.A. and (1,14) F.A. bearing a nitroxide paramagnetic group respectively at the fifth carbon (i.e. near the polar moiety) and the 16^{th} carbon (i.e. near the terminal methyl group) of the hydrophobic chain were tested for quenching of the emission fluorescence intensity of anilinonaphtyl spectrin. Results are shown in figure 1 on a semilogarithmic plot. Both fatty acids quench completely the fluorescence of the anilinonaphtyl group in the 10^{-4} M range. These low concentrations

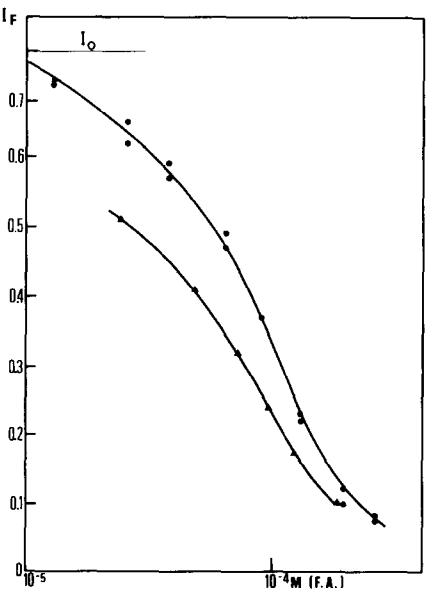


Figure 1. Quenching of the fluorescence intensity of anilinonaphtyl spectrin by nitroxide analogs of fatty acids : (10,3) F.A. (●) and (1,14) F.A. (▲). Values plotted are the intensities at 438 nm (the maximum emission wavelength) in arbitrary units. Io corresponds to the fluorescence intensity in the absence of fatty acid analogs.

of paramagnetic probe allow the elimination of a pure collisional quenching mechanism and infer an affinity of fatty acids towards spectrin (14). Moreover, the sigmoidal curves obtained are characteristic of a binding process and the values of the affinity constants can be deduced from the 50 % quenching concentrations of fatty acid analogs : $Ka_{(1,14)} = 1.85 \times 10^4\ M^{-1}$ and $Ka_{(10,3)} = 1.1 \times 10^4\ M^{-1}$.

Binding of spin labeled phosphatidylserine and phosphatidylcholine

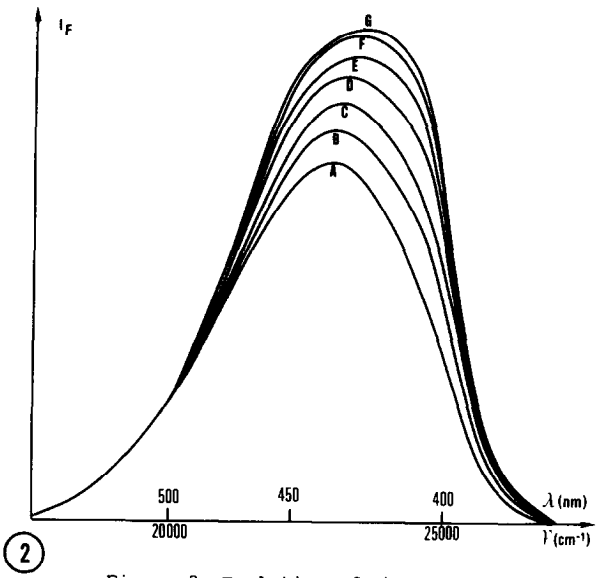
Quenching of spectrin fluorescence by phospholipid analogs bearing a five carbon β-chain substituted with a nitroxide was then measured. Two analogs differing only in the nature of their polar end (serine or choline) were tested : (0,2) P.S. and (0,2) P.C. While (0,2) P.C. had no effect on the fluorescence spectrum of anilinonaphtyl spectrin, (0,2) P.S. added in the micromolar range causes a decrease of fluorescence intensity of the order of 30 % with an apparent affinity of $2 \times 10^5\ M^{-1}$. It must be mentioned here that the micelle formation by (0,2) P.S. at the higher concentrations used precludes a precise evaluation of the binding constant. Nevertheless these experiments show that spectrin clearly exhibits specific binding sites for negative phospholipids.

Interaction with phospholipid vesicles

Two kinds of lipids were tried : egg yolk phosphatidylcholine and phosphatidylserine derived from egg phosphatidylcholine by a transphosphatidylation catalyzed by phospholipase D so that they differ only by their polar end.

Sonicated vesicles giving rise to low light scattering were progressively added to anilinonaphtyl spectrin up to a ratio of 1 dimer to 4000 lipid at 30 °C. Phosphatidylcholine vesicles caused no change in the fluorescence emission spectrum of spectrin. Conversely, in the presence of phosphatidylserine vesicles the emission maximum slowly exhibits a blueshift associated with an increase of fluorescence intensity as shown in figure 2 which gives the emission spectra ($I_F = f\ (1/\lambda)$) at diverse phosphatidylserine concentrations. This phenomenon is saturable and the final spectrum found has its maximum emission wavelength shifted to 424 nm and a maximum intensity increased by about 40 %.

Figure 3 represents the relative variation of the fluorescence quantum yield, deduced from integration of the spectra of figure 2, with the phosphatidylserine to spectrin ratio. From the quantum yield variation, a scatchard plot can be drawn assuming that saturation corresponds to the quantitative binding of spectrin to phosphatidylserine. The result is shown in Figure 4 : spectrin binds to phosphatidylserine vesicles at a ratio of one dimer to 1500 phospholipids and with an affinity of about $3 \times 10^7\ M^{-1}$.



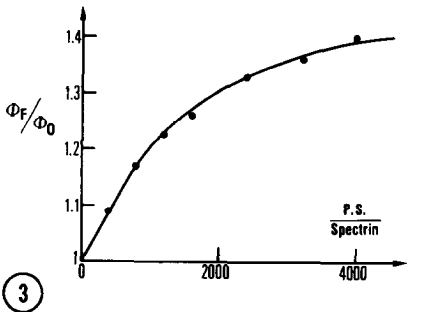


Figure 2. Evolution of the fluorescence emission spectrum of anilinonaphtyl spectrin in the presence of phosphatidylserine vesicles. A : anilinonaphtyl spectrin alone at 2.10^{-7} M ; B : with 0.095 mM ; C : with 0.185 mM ; D : with 0.36 mM ; E : with 0.53 mM ; F : with 0.69 mM and G : with 0.85 mM - 1 mM (at saturation) phosphatidylserine.

Figure 3. Relative quantum yield variation of anilinonaphtylspectrin versus phosphatidylserine concentration as expressed as the ratio of phosphatidylserine to spectrin concentrations.

Vesicles composed of 80 % phosphatidylserine and 20 % (1,14) P.C. - a phosphatidylcholine analog spin labeled at the 16th carbon of the β hydrocarbon chain - were also tested : they caused a 15 % quenching of

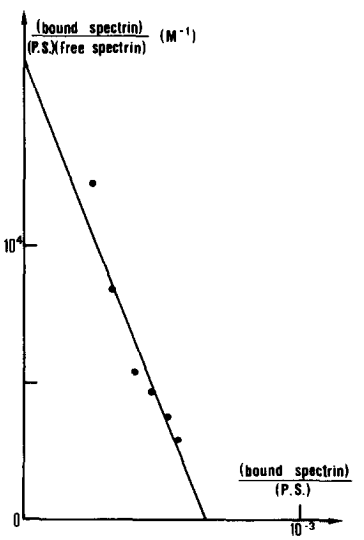


Figure 4. Scatchard representation deduced from figure 3 assuming that total binding of spectrin to phosphatidylserine vesicles occurs at saturatio

anilinonaphtylspectrin fluorescence compared to the intensity in the presence of phosphatidylserine vesicles containing the same proportion of egg phosphatidylcholine. The complete inclusion of (1,14) P.C. into the vesicles was checked by E.P.R. (data not shown).

DISCUSSION

The emission spectrum of the anilinonaphtyl labeled spectrin outlines the presence of hydrophobic sites that possess a reactive function towards maleimides (thiol or amine). At present, their distribution among the 8 labeled sites cannot be precised since the fluorescence intensity dramatically decreases with polarity but the hydrophobic character of anilinonaphtyl-maleimide certainly strongly favours the labeling of such sites.

The quenching of emission fluorescence intensity by spin-labeled lipids in solution in the 10^{-4} M range for fatty acids and 10^{-6} M range for phosphotidylserine shows that spectrin is able to bind these molecules at sites that overlap the hydrophobic anilinonaphtyl sites. Moreover, these experiments demonstrate a high specificity of spectrin towards phosphatidyl-serine already noted by other authors (1). This specificity is further confirmed by our results on the interaction of spectrin with phospholipid vesicles since the phospholipids used differ only in the nature of their polar end, the recognition process certainly involves binding sites for the serine moiety. On the other hand, the changes in the fluorescence spectrum of the anilinonaphtyl groups that occur in the presence of phosphatidylserine vesicles are evidence that the probe is in a more hydrophobic environment which suggests its penetration into the bilayer. Moreover, this hypothesis is in accordance with the observed quenching of fluorescence caused by the presence of (1,14) P.C. within lipid vesicles.

The great length of spectrin (100 nm per heterodimer) makes it the best candidate for a stabilization of the plasma membrane via cross-linking of intrinsic membrane proteins or by direct interaction with phospholipids. The forementioned experiments tend to outline the relevance of the second possibility : spectrin is shown to interact quite selectively with phosphatidylserine, a phospholipid exclusively located in the inner layer of the erythrocyte membrane and therefore readily accessible for binding to spectrin molecules. Future work should determine the penetration depth of the probes into the bilayer and their location within the spectrin molecule .

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REFERENCES

1. Haest, C.W.M. (1982) Biochim. Biophys. Acta 694, 331-352.
2. Hargreaves, W.R., Giedd, K.N., Verkleij, A. and Branton, D. (1980) J. Biol. Chem. 255, 11965-11972.
3. Tyler, J.M., Hargreaves, W.R. and Branton, D. (1979) Proc. Nat. Acad. Sci. USA 76, 5192-5196.
4. Isenberg, H., Kenna, J.G., Green, N.M. and Gratzer, W.B. (1981) FEBS Lett. 129, 109-112.
5. Mombers, C., De Gier, J., Demel, R.A. and Van Deenen, L.L.M. (1980) Biochim. Biophys. Acta 603, 52-62.
6. London, E. (1982) Mol. Cell. Biochem. 45, 181-188.
7. Ungewickell, E. and Gratzer, W. (1978) Eur. J. Biochem. 88, 379-385.
8. Laemmli, U.K. (1970) Nature (London) 227, 680-685.
9. Singleton, W.S., Gray, M.S., Brown, H.L. and White, J.L. (1965) J. Am. Chem. Soc. 42, 53-56.
10. Comfurius P. and Zwaal, R.F.A. (1977) Biochim. Biophys. Acta 488, 36-42.
11. Hubbel, W.L. and Mc Connell, H.N. (1971) J. Am. Chem. Soc. 93, 314-326.
12. Seigneuret, N. and Devaux P. (submitted).
13. Kanaoka, Y., Machida, M., Machida, M. and Sekine, T. (1973) Biochim. Biophys. Acta 317, 563-568.
14. Singer, L.A. and Davis, G.A. (1967) J. Amer. Chem. Soc. 89, 158-159.