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Effect of sealing on the incorporation of pyrene in goat erythrocyte ghosts – A fluorescence study

P. NANDY★, M. DUTTA CHOUDHURY and P. CHAKRABARTI
Department of Chemistry, Bose Institute, 93/1, Acharya Prafulla Chandra Road, Calcutta 700 009.

★ Department of Physics, Jadavpur University, Calcutta 700 032

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Abstract. The incorporation of pyrene within the membrane interior of goat erythrocyte ghost has been estimated from its fluorescence spectrum. The excimer to monomer fluorescence intensity ratio of embedded pyrene is a function of the fluidity of its environment and the magnitude of its incorporation. Our study shows that this ratio is considerably less (30%) in a pre-sealed ghost than in the non-sealed ghost revealing that the site of incorporation of the probe is indeed the hydrophobic interior of the membrane; as in the later case, the probe has access to the membrane interior from both sides of the membrane. Our study on kinetics of molecular exchange indicates a very fast (of the order of seconds) transfer rate of pyrene from probed to unprobed erythrocyte ghosts through the aqueous phase rather than actual fusion of the membranes.

Keywords. Erythrocyte ghosts; probe incorporation; excimer fluorescence.

Introduction

Spectroscopic probes have long been used in the development of our knowledge of cells and membranes (Vanderkooi and Callis, 1974; Galla and Sackman, 1974; Morrisett *et al.,* 1975; Dembo *et al.,* 1979; Georgescauld *et al.,* 1979; Flamm *et al.,* 1982). Probes of different polarity can be incorporated in different parts of the membrane. We have used pyrene, an excimer forming fluorescent probe, to study the fluidity picture within the goat erythrocyte ghost membrane. The probe pyrene has already been used by several workers both in model membranes as well as in biological membranes (Galla and Sackman, 1974; Morrisett *et al.,*1975; Dembo *et al.,* 1979; Georgescauld *et al.,* 1979; Flamm *et al.,* 1982; Sengupta *et al.,* 1976, 1982). The single most important characteristic feature of this lipophilic, non-ionic probe pyrene is its ability to form short-lived dimers in the excited state. The ratio of the monomer (I at λ = 390 nm) to excimer intensity (I′ at λ'=485 nm) of the embedded pyrene when illuminated at 320 nm, is an index of fluidity parameter of its environment and also of its magnitude of incorporation (Galla and Sackman, 1974).

Pyrene being apolar in nature, its natural location is in the hydrophobic lipid region of the membrane. In our study with the goat erythrocyte ghosts, it is observed that the probe incorporation is more in the non-sealed ghost compared to that in the pre-sealed ghost This implies that the probe has access both to the outer and inner leaflets of the erythrocyte membrane in case of the non-sealed ghost whereas for the pre-sealed ghost only the outer leaflet is accessible. The kinetics of

mixing between probed and unprobed ghosts reveal a fast transfer of probe rather than actual fusion of the membranes, which is supposed to be a slow process (Sengupta *et al.,* 1976).

Materials and methods

Glass distilled water, oxygen-free buffer, zone-refined pyrene, analytical-grade chemicals and spectral grade solvent had been used. Fluorescence measurement was made with Carl-Zeiss spectrofluorometer (model No. ZFM/4C). Sample temperature was kept constant (±0.5°C) by circulating water at a particular temperature through the sample holder chamber of the spectrofluorometer.

Fresh blood from goat was collected from the local market in 168 mM NaCl solution (normal saline) with 0.35% sodium citrate as anticoagulant.

Isolation of erythrocyte ghost

Goat blood sample was centrifuged at 1000 *g* for 20 min. Buffy coat was removed by aspiration and by resuspending the pellet three times in normal saline and centrifuging at 1000 *g* for 20 min. The pellet which contained only red blood cells was then haemolysed by adding 50-60 times volume of 10 mM NaCl solution at 0°C. Good frothing indicated completion of haemolysis. Cell debris was removed by centrifuging at 1000 *g* for 20 min and the pellet was discarded. The supernatant was centrifuged at 20,000 *g* for 40 min. The pellet was washed two more times in 10 mM NaCl solution. The pink jelly-like pellet contained non-sealed ghost (Marchesi, 1974). All the operations were carried out at 4°C. Protein content was estimated by the method of Lowry *et al.* (1951) .

For sealing, to 1 ml of non-sealed ghost suspension in 10 M NaCl solution, 0.78 ml of 1 mM NaCl solution was added. The solution was then stirred for 15 min at 0°C, to give sealed ghost in 168 mM NaCl solution (Zwaal *et al.,* 1975).

Incorporation of pyrene

Using various concentrations of pyrene and different times and temperature of incubation, it was found that the optimum condition for pyrene incorporation involved incubation with 0.3 mg pyrene/5 mg of protein at 37°C for 2 h (data not shown). A thin film of pyrene (0.3 mg) was made at the bottom of a round bottom flask by spreading its chloroformic solution and then evaporating the solvent under nitrogen atmosphere (Morrisett *et al.,* 1975). The probe was incorporated as follows:

(a) *In non-sealed ghost:* 5 ml of non-sealed ghost suspension containing 5 mg of protein in 10 mM NaCl solution was added to the pyrene film. The mixture was then incubated by stirring constantly at 37°C for 2 h. Unbound pyrene was removed by centrifugation at 1000–2000 *g,* followed by dialysis. Supernatant was used for fluorescence measurement.

(b) *In pre-sealed ghost:* 5 ml of the pre-sealed ghost suspension containing 5 mg of protein in normal saline was added to the pyrene film. The rest of the procedure was as above.

Pyrene exchange study

Equal volumes of pyrene-probed and unprobed sealed ghost suspension were mixed and the fluorescence spectrum was taken immediately after mixing. The peak intensities were monitored at intervals of 1, 2, 5 and 10 min. The whole spectrum was taken once again after a steady state had been reached. The experiment was carried out at 37°C.

Results

Pyrene-incorporated membrane preparation, when illuminated at 320 nm, shows a highly resolved monomer of intensity I′ at 390 nm and a broad excimer emmission band of intensity I′ at 485 nm (figure 1a). The excimer formation being

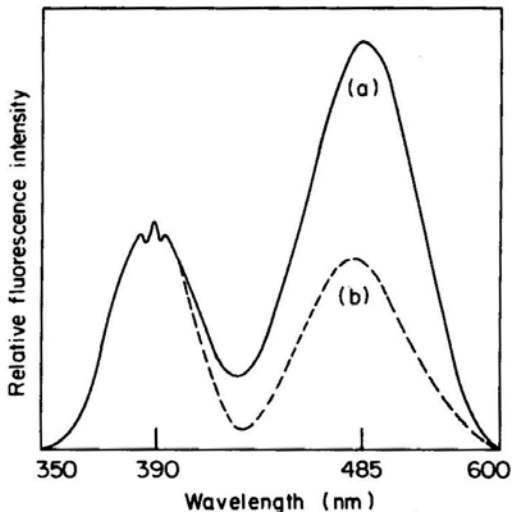

Figure 1. Kinetics of probe transfer from probed to unprobed sealed erythrocyte ghosts. Sample temperature 37°C.

(a) Spectrum of pyrene-probed erythrocyte sealed ghost before mixing. I′/I = 1.8.
(b) Spectrum of the mixture of equal volumes of probed and unprobed sealed erythrocyte ghost immediately after mixing.
I′/I = 0. 89, value remains unchanged for successive readings at 1, 2, 5 and 10 min intervals (only the peak intensities were monitored).

a diffusion-controlled process, this ratio I'/I is an indicator of the fluidity picture of its environment. At a constant temperature, however, the ratio is directly proportional to the effective concentration of the probe incorporated within the membrane. Comparison of I′/I for pre-sealed ghost and non-sealed ghost shows that pyrene incorporation in the previous case is about 30% less than that in the later (I′/I=1.8 for pre-sealed ghost and 2.33 for non-sealed ghost).

The kinetics of molecular exchange between ghost membranes have been studied by rapid mixing of equal volumes of pyrene-probed and unprobed sealed ghost membrane. I′/I reduces to half of its original value immediately after mixing (figure 1b) and remains unchanged upto intervals of. 1, 2, 5 and 10 min.

Discussion

Pyrene being non polar, its natural site of incorporation will be the lipid mileau of the membrane. The comparison of its incorporation in sealed and non-sealed ghosts shows that this indeed is the situation. Pyrene approaches the hydrophobic interior both from inside and outside the membrane surface in case of non-sealed ghost, whereas it has access only from outside in case of pre-sealed ghost. Result shows that incorporation in non-sealed ghost is indeed 30% more than that in the sealed ghost.

It has been shown by several workers (Sengupta *et al.,* 1976; Thilo, 1977) that label exchange between phospholipid bilayers is a fast process and that actual fusion, which is a very slow process takes place only under special condition. In our kinetics study, we verify that rapid transfer of probe takes place between probed and unprobed sealed erythrocyte ghost membrane. The ratio I′/I of the mixture of equal volumes of probed and unprobed membrane reduces to half of its original value immediately after mixing remains unchanged. This indicates a very rapid transfer of the membrane bound pyrene.

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