Distribution of Potential-Sensitive Probe diS-C_3-(5) in Membrane Suspensions

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Abstract. Based on analysis of fluorometric titration curves a quantitative method for evaluating a potential-sensitive probe of partition constants between aqueous and lipid phases is proposed. The partition constants of the probe DiS-C_3-(5) in sarcoplasmic reticulum (SR) vesicle suspensions were estimated to be $(1.15 \pm 0.04) \times 10^7$ and $(6.12 \pm 0.07) \times 10^6$ at low (0,3 mol/l sucrose) and high (0,15 mol/l KCl) ionic strength respectively. For azolectin liposome suspension at high ionic strengh this parameter was $(3.4 \pm 0.2) \times 10^7$.

Key words: Sarcoplasmic reticulum — Azolectin liposomes — Potential-sensitive probe diS-C_3-(5) — Partition constants — Membrane potential

Introduction

In recent years different potential-sensitive fluorescent probes received wide application in studying the electrical activity of biological membranes (Waggoner 1979; Cohen and Salzberg 1978; Bashford and Smith 1979). A great number of works is devoted to study the relationship between the optical response of the probe and the changes in transmembrane potential (Sims et al. 1974; Waggoner et al. 1977; Loew et al. 1978; Smith et al. 1980). The investigations show that the behaviour of the probes in a membrane system is determined by both their structure and the structural peculiarities of the membrane under study. There are probably no reasons to suggest the existence of a common mechanism of potential-dependent response for all objects and all types of the probes studied. That is why the experimental procedure and interpretation of the findings must be performed with great care. In order to clarify the mechanism of the potential-dependent response of a probe it is necessary, first, to study the equilibrium partition of the probe between the membrane and the aqueous phase, and then between monomers and aggregates in water and membranes.

The partition constant is the most convenient parameter to describe the equilibrium interphasic distribution (Tanford 1973):

$$K = X_p^m / X_p^a = e^{-\Delta\mu/RT}$$

where X_p^m and X_p^a are molar parts of the dye in the membrane and aqueous phase respectively, $\Delta\mu$ is the chemical potential difference of the probe in membranes and in water, R is the univesal gas constant and T is the absolute temperature. In this work we have calculated the constant for the probe 3,3′-dipropyl-2,2′-thiadicarbocyanin (diS-C_3-(5)) in the suspension of SR vesicle membranes and in salt medium for liposomes from total soybean phospholipids (azolectin).

When estimating the constant of the probe partition in SR membrane suspension we have considered as a membrane "phase" only the lipid component of the vesicles. This is not only because azolectine liposome suspension as a system was chosen for comparison but also because the asumption about the probe-membrane interaction was made. Two hydrophobic chains of the probe must play an important role in the probe-lipid interaction. This is evident by the

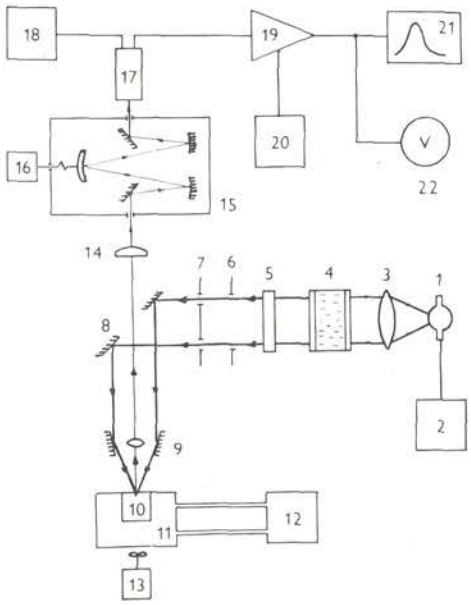

Fig. 1. Block-scheme of a unique spectrofluorometer with registration of fluorescence from the front surface of the sample. 1 — high pressure mercury arc lamp DRSh-250-2, 250 W; 2 — stabilized DC power supply of the mercury arc lamp; 3 — condenser quartz lens; 4 — heat absorption filter with quartz windows; 5 — changeable interference filters 6 — with HBW 12 nm; 7 — diaphragmes; 8 — annular mirror; 9 — epiobjective 21 × 0,40; 10 — quartz sample cuvette; 11 — thermostated cuvette holder; 12 — ultrathermostate U I; 13 — magnetic stirrer; 14 — cylindrical lens; 15 — monochromator; 16 — driver of monochromator; 17 — photomultiplier; 18 — photomultiplier power supply; 19 — preamplifier; 20 — preamplifier power supply; 21 — recorder "Watanabe" (Japan); 22 — digital voltmeter F 204/1.

absence of any interaction between the membranes and hydrophilic analogs of diS-C_3-(5) in which one proton of the end methyl group is replaced by the SO_3^--group (Sims et al. 1974).

Materials and Methods

SR vesicles were isolated from rabbit white skeletal muscles according to Ritov et al. (1977). The protein concentration was determined according to Lowry et al. (1951) in the presence of 1% desoxycholate. The preparation was kept at 0°C and used during 5 days.

To prepare liposomes 10% alcohol solution of lecithin-standard and azolectin ("Sigma"), preliminary purified with acetone, was used. The desoxycholate ("Spofa", recrystallized) solution of soybean lipids (azolectin) or lecithin was sonicated in salt medium (0.075 mol/l K_2SO_4, pH 7.2) with ultrasound dispergator UZDN-2T for 10 min till the solution was clarified. To remove the detergent, the obtained liposome suspension was dialyzed against the buffer, containing 0.075 mol/l K_2SO_4, 10 mmol/l Tris-H_2SO_4, pH 7.2 during 24 hr (Szoka 1980).

The probe diS-C_3-(5) was used. The absorption spectra of the dye were registered with "Specord UV VIS".

The fluorescent spectra were measured with a unique spectrofluorometer, the sample being excited through interference filters and an epiobjective. The fluorescence was registered from the front surface of the sample. The constructional features of the device made it possible to measure the fluorescence of a thin layer (less than 1 mm) of the solution without spectrum distortions due to the effects of the intrinsic filter and light scattering (Vladimirov and Dobretsov 1980).

The fluorescence was excited with the radiation of a mercury lamp through an interference filter with $\lambda_{exc} = 580$ nm, with HBW 12 nm, and was registered with an emission slit of 5 nm. The measurements were made at room temperature. The block-scheme of the device is shown in Fig. 1.

Results and Discussion

Spectral characteristics of diS-C_3-(5)

The spectrum parameters of this probe are presented in the literature (Sims et al. 1974; Hladky and Rink 1976; Tsien and Hladky 1976; Pechatnikov et al. 1979), so only some notices essential for understanding its behaviour in different media are given in this paper.

First of all, it should be noted that in sucrose medium the probe remains mainly in a monomeric form up to a concentration of 10^{-5} mol/l. It is confirmed by a linear dependence of optical density ($\lambda_a = 647$ nm) and fluorescence intensity ($\lambda_f = 675$ nm) on the dye concentration. The probe molecules are charged positively at neutral pH. So, different anions (in particular Cl^-) decreasing electrostatic repulsion increase the aggregation of molecules in water medium. The dependence of diS-C_3-(5) absorption spectra on KCl concentration is shown in Fig. 2. It is seen, that 10^{-1} mol/l KCl induces a marked aggregation of the dye molecules. The appearance of the absorption band in the range more than 700 nm points to this fact.

The analysis of the excitation spectra of the probe shows that at $\lambda_f = 670$ nm only dye monomers fluoresce both in water and membranes (Sims et al. 1974). The

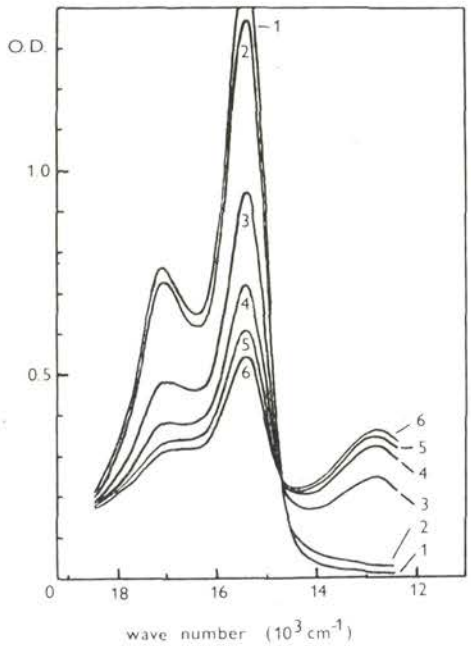

Fig. 2. The effect of KCl on the absorption spectrum of the probe in the medium, containing 0.3 mol/l sucrose, 5×10^{-3} mol/l imidazole, pH 7.0: 1 — without KCl additions; 2 — 0.05 mol/l KCl; 3 — 0.1 mol/l KCl; 4 — 0.15 mol/l KCl; 5 — 0.2 mol/l KCl; 6 — 0.25 mol/l KCl.

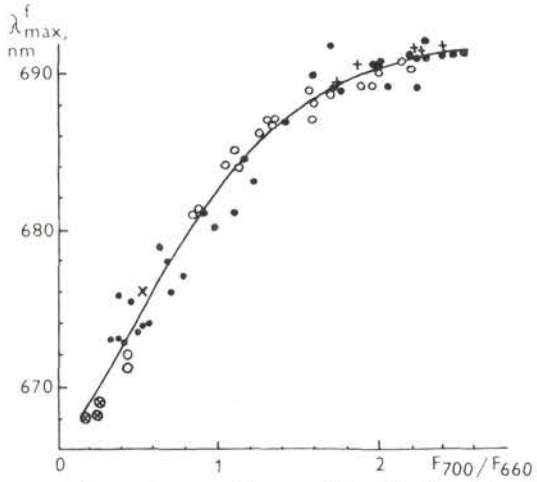

Fig. 3. The correlation between the spectrum maximum position (λ^{f}_{max}) and the ratio of the intensities (F_{700}/F_{660}) on the slopes of this spectrum at variable molar ratios of diS-C_3-(5) and the membranes in the solution: lecithin liposomes — o; azolectin liposomes — +; SR vesicles — ●; in the buffer medium without the membranes — ⊗. Ethanol solution of diS-C_3(5) is marked with × molar ratios of the probes and for SR vesicles are shown in Fig. 4, 5).

fluorescent spectrum of the membrane monomers shifts to the red by about 20 nm compared to that of water solution. An analogous long-wave shift of the fluorescence spectrum is observed for the solvents less polar than water (Sims et al. 1974; Hladky and Rink 1976; Tsien and Hladky 1976; Pechatnikov et al. 1979).

The ratio of the intensities at the spectrum slopes (F_{700}/F_{660}) is a convenient parameter for registration of dye fluorescent spectrum shifts. The correlation between the maximum of the diS-C_3-(5) fluorescent spectrum and the F_{700}/F_{660} parameter at different probe and membrane concentrations is shown in Fig. 3. Fluorescence spectra were not corrected.

Partition of diS-C_3-(5) between medium and membranes

The curves of fluorometric titration of the probe solution (at different initial concentrations) by SR membrane suspension are given in Fig. 4 (A, B, C). The abscissa presents the total quantity of the lipids in the sample (in mol/l) taking into consideration that preparations contain about 0.5 mg lipids per 1 mg protein (Ritov 1977). The average molecular weight of membrane phospholipids is taken to be 750. The intensity of fluorescence F_{660} ($\lambda_f = 660$ nm) falls rapidly down even at small membrane concentrations, whereas the position of the spectrum maximum does not pracically change (curves 1 and 3 in Fig. 4). The subsequent additions of

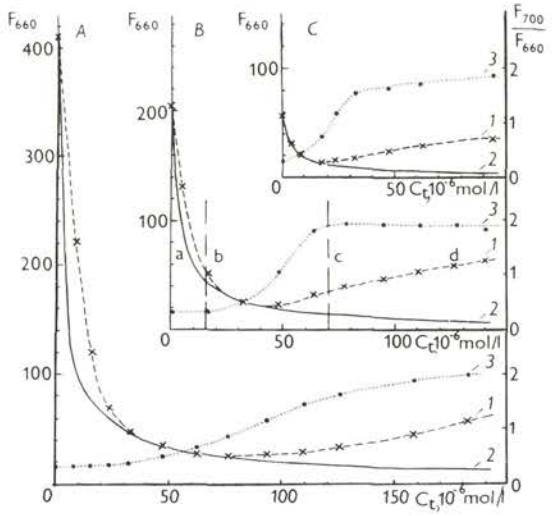

Fig. 4. Fluorometric curves of diS-C_3-(5) titration by SR membranes in the medium, containing 0,3 mol/l sucrose, 10^{-2} mol/l imidazole-MOPS, pH 7,0.F_{660} is fluorescence intensity at $\lambda_f = 660$ nm in the relative units. Fixed concentration of diS-C_3-(5): A — 5.4×10^{-6} mol/l; B — 2.7×10^{-6} mol/l; C — 0.9×10^{-6} mol/l. 1 — experimental titration curve; 2 — calculated dependence of the quantity of monomers in water on the amount of lipids added; 3 — the change of maximum position of the fluorescence spectrum. Ordinate for curves 1 and 2 is in the left, and for curve 3 is in the right.

the membranes cause less effective fluorescence quenching and, at last, the intensity gradually increases. Since neither dimers nor aggregates in this range of the spectrum contribute to the fluorescence, the titration curves reflect the monomer partition between water and membranes, on one hand, and between monomers and aggregates on the other. The fluorometric titration curves may be devided into three parts (Fig. 4, B). The first part (ab) shows the fluorescence quenching without spectrum shift. In this case all the probe molecules in the membranes are apparently in the aggregate state and the total fluorescence is exclusively determined by "aqueous" monomers. The second part (bc) reflects the gradual increase of the "membrane" monomer contribution. This process induces a long-wave shift of the fluorescent spectrum. The third part (cd) reflects further increase of the membrane monomer fluorescence and is characterized by a gradual change to a plateaul.In this case the spectrum does not practically shift, because the contribution of aqueous monomers becomes negligible at rather high membrane concentrations.

Analogous curves were obtained both for SR vesicles (Fig. 5, A, B, curves 1 and 3), and for the liposomes from azolectin (not shown) in salt medium (0.15 mol/l KCl and 0,075 mol/l K_2SO_4, consequently). The partition constant may be calculated from the first part of the titration curve (curve 1) because the total fluorescence at $\lambda = 660$ nm is determined by the monomeric form of the dye in water. It is possible to calculate the aqueous monomer fluorescence contribution to the remaining part of the titration curve using the partition constants and thus the probe contents for the medium and membranes.

It should be noted that at high initial probe concentrations the calculated distribution curves of the first part do not always coincide with the experimental ones (Fig. 4, A, B). At high partition constants all the amount of the probe cannot

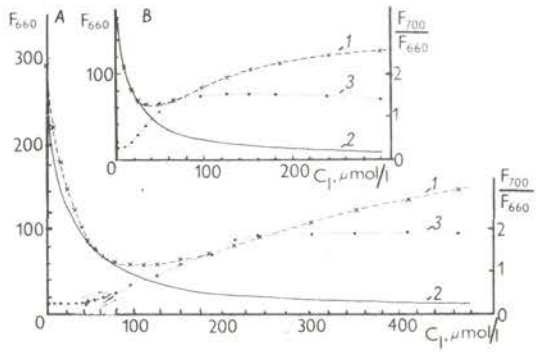

Fig. 5. Fluorometric curves of diS-C_3-(5) titration by SR membranes in the medium, containing 0.15 mol/l KCl, 10^{-2} mol/l imidazole-MOPS, pH 7.0. diS-C_3(5) concentrations: A – – 1.9×10^{-6} mol/l, B — 5.4×10^{-6} mol/l. (For explanation of the curves see Fig. 4).

be taken up with the membranes added and there is a linear dependence of the fluorescence intensity on the membrane concentration. The less the initial probe concentration, the more the theoretical curve is similar to the experiment titration curve (Fig. 4, A, B, C, curves 1 and 2).

While calculating the partition constant for the salt medium the probe concentration was estimated from the calibrating curve (the concentration dependence of probe fluorescence intensity in a medium without membranes).

The quantitative dye distribution may be described by a partition coefficient K'_p, i. e. by the ratio of molar concentration of the probe in the aqueous and lipid phase. In this case K'_p is related to the partition constant like this: $K'_p = K \cdot V_a / V_l$, where V_a and V_l are molar volumes of water and lipid, respectively. This formula is valid, if $n_p^m \ll n_l$ and $n_p^a \ll n_a$. Taking into consideration that the partial specific density of lipid, as water density, is approximately 1 g/cm^3, we took $V_a = 18 \times 10^{-3}$ l, $V_l = 0.750$ l, therefore $K'_p \simeq 0.024$ K.

The partition constant and partition coefficient values in SR membranes and azolectin liposomes are shown in Table 1. It is seen from Table 1, that for SR

Table 1. The values of partition constants and coefficients in membrane suspensions

Object, experimental conditions	K	K'_p
1. SR vesicles in the medium: 0,3 mol/l sucrose, 10^{-2} mol/l imidazole — MOPS, pH 7,0; initial concentration (C_o) of diS-C_3(5):		
0.90×10^{-6} mol/l	1.12×10^7	2.7×10^5
2.7×10^{-6} mol/l	1.12×10^7	2.7×10^5
5.4×10^{-6} mol/l	1.2×10^7	2.9×10^5
	$(1.15 \pm 0.04) \times 10^7$	$(2.76 \pm 0.09) \times 10^5$
2. SR vesicles in the medium: 0.15 mol/l KCl, the same buffer,		
$C_o = 1.9 \times 10^{-6}$ mol/l	6.2×10^6	1.5×10^5
5.4×10^{-6} mol/l	6.05×10^6	1.4×10^5
	$(6.12 \pm 0{,}07) \times 10^6$	$(1.45 \pm 0.05) \times 10^5$
3. Liposomes from azolectin in the medium: 0.075 mol/l K_2SO_4, 10^{-2} mol/l tris-H_2SO_4, pH 7.2		
$C_o = 10^{-6}$ mol/l	3.7×10^7	8.9×10^5
10^{-6} mol/l	3.1×10^7	7.4×10^5
2×10^{-6} mol/l	3.5×10^7	8.4×10^5
2×10^{-6} mol/l	3.3×10^7	7.9×10^5
	$(3.4 \pm 0.2) \times 10^7$	$(8.15 \pm 0.47) \times 10^5$

vesicles in salt medium (0.15 mol/l KCl) the partition constant is about two times less than for those in sucrose medium. It may be both due to a decrease of electrostatic interaction between the oppositely charged probe molecules and membranes at a partial compensation of the charge by counterions and due to some decrease of the chemical potential of the probe in water. The later effect is determined by a decrease of electrostatic repulsion between the probe molecules and by a reduction of the total hydrophobic surface of the molecule due to dimerization.

For azolectin liposomes, the partition constant is about 6 times higher than for the lipid component of SR membranes. We suggested that the probe cannot interact with the boundary lipids, immobilized by protein-lipid interaction.

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Appendix

The calculation of the constant of dye partition between aqueous and membrane phase.

Let us consider the constant of the partition between two phases — the aqueous and lipid component of the membrane suspension:

$$K = X_p^m / X_p^a \tag{1}$$

where, X_p^m and X_p^a are molar portions of the dye in the agueous and membrane phase. They are respectively equal to:

$$\begin{aligned} X_p^m &= n_p^m/(n_p^m + n_l) \\ X^a &= n_p^a/(n_p^a + n_a) \end{aligned} \tag{2}$$

where, n_l and n_a are the number of lipid and water molecules in the membrane and aqueous phases, and n_p^m and n_p^a are the number of probe molecules in the membranes and water. According to (1) and (2)

$$\begin{aligned} n_p^m &= n_p^o - x \\ n_p^m(x + n_a) &= K.x(n_p^m + n_l) \end{aligned} \tag{3}$$

where x is n_p^a and n_p^o is the total quantity of the probe molecules in the suspension. The system of two equations relates x to a variable value n_l (titration by lipids).

Thus,

$$(K-1)x^2-[(K-1)n_p^o+n_a+Kn_l]x+n_p^o n_a=0 \tag{4}$$

In this case:

$$x_{1,2}=\frac{K.n_l+\beta\pm\sqrt{(K.n_l+\beta)^2-4(K-1)\alpha}}{2(K-1)} \tag{5}$$

where

$$\alpha=n_p^o.n_a,\ \beta=n_a+(K-1)n_p^o$$

To choose the sign before the square root, let us take $x=n_p^o$ at $n_l=0$. In this case:

$$x_{1,2}^o=\frac{\beta\pm\sqrt{\beta^2-4(K-1)\alpha}}{2(K-1)} \tag{6}$$

The expression under the square root:

$$n_a^2-2(K-1)n_p^o.n_a+[(K-1)n_p^o]^2$$

is a difference squared, therefore, it is necessary to analyse the ratio between n_a and $(K-1)n_p^o$.

It follows from the second equation (3) that at $n_l\to 0$

$$Kn_p^o=\xi(n_p^o+n_a),$$

where

$$\xi=n_p^m/(n_p^m+n_l)<1$$

This expression can be changed

$$n_p^o(K/(\xi-1))=n_a \qquad \text{i. e}$$

$n_p^o(K-1)$ is less than n_a, as $\xi<1$. Thus, the radicand looks like:

$$[n_a-(K-1)n_p^o]^2.$$

Therefore,

$$x_{1,2}^o=\frac{n_a+(K-1)n_p^o\pm[n_a-(K-1)n_p^o]}{2(K-1)} \tag{7}$$

and it is necessary to choose the minus for $x^o=n_p^o$. In (5) n_p^o, n_a, and n_l are given by the conditions of our experiment in which a value x proportional to fluorescence intensity is measured:

$$x=\frac{K.n_l+\beta-\sqrt{(K.n_l+\beta)^2-4(K-1)\alpha}}{2(K-1)} \tag{8}$$