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QUANTITATION OF THE FÖRSTER ENERGY TRANSFER FOR TWO-DIMENSIONAL SYSTEMS. II. PROTEIN DISTRIBUTION AND AGGREGATION STATE IN BIOLOGICAL MEMBRANES

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Analytical solutions are presented of the average rate of the Förster energy transfer for several processes affecting intrinsic membrane proteins within a phospholipid bilayer. The physical phenomena considered here are: lateral phase separation of the protein, i.e., formation of eutectic mixtures, changes in the aggregation state of the protein and non-random distribution of protein molecules. It is shown that the average rate of energy transfer among protein and phospholipid molecules labelled with donor and acceptor molecules, respectively, allows differentiation between them and also that the average rate of energy transfer can be used to quantitate these phenomena.

1. Introduction

Although much research effort has been devoted to the understanding of intrinsic membrane protein regulation [1–3], this is still a controversial subject (see for example refs. [4–6]). Such a situation can be seen as a consequence of the great complexity of physical phenomena taking place simultaneously in biological membranes and affecting the functional efficiency of their protein components. So, melting processes and phase separation of phospholipids appear to affect intrinsic protein phase separation, i.e., the formation of eutectic lipid-protein mixtures [4,7], protein aggregation state [1,4] and asymmetric incorporation of the protein into the membrane [8], and probably control, via different simultaneous operating mechanisms, the functionality of intrinsic membrane proteins in a very sophisticated way. On the other hand, this situation is further complicated due to the difficulties in gaining a deeper knowledge of all these phenomena under experimental conditions similar to those used to measure the functional properties of these membrane-bound proteins.

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In this paper and continuing the study developed in the preceding paper [9], it is shown that Förster energy transfer can be used to quantitate several relevant processes involving intrinsic membrane proteins. These are: eutectic lipid-protein mixtures, non-random lateral protein distribution and changes in the aggregation state of the protein for simple cases, such as alteration of the protein aggregation state due to monomer = dimer and monomer = dimer = tetramer equilibrium displacements. Special emphasis is placed on differentiation between the effects of each of these processes on the rate of energy transfer because of the evident interest in determining their relative roles in many problems of membrane biology.

2. Preliminary remarks

It should be pointed out that the average donor-acceptor distance determined from energy transfer measurements and those referred to in the following text are not only an average of all the distances in the bilayer of donor-acceptor pairs of protein molecules and of protein-phospholipid molecules, but are also an average of all the possible orientations of donor and acceptor fluorophores [10]. The latter factor probably needs a more detailed discussion. First of all, if both donor

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and acceptor are attached to the polar part of the protein, i.e., the protein region not embedded in the lipid bilayer, they can be assumed to be freely rotating with respect to each other, and then, from time and space averaging, an orientation factor of $K^2 = \frac{2}{3}$ follows. The error in the determined distances arising from such an assumption can be neglected if one considers the relatively small size of the fluorophore when compared to that of the protein. However, another orientation factor, K_p, must be considered. This factor concerns the relative orientation of two protein molecules due to the fixed position of the label(s) in the protein molecules. It can be seen that the fluorescent label will probably not be bound to the center of the protein region emerging from the bilayer. The time-averaged energy transfer over all the possible pairs of protein molecules in the bilayer must take this factor into account. The assumption $K_p^2 = \frac{2}{3}$ can be made again but further analysis is required to estimate the real distance, r_p. For this one has to take into account that only part of the protein surface is exposed to the aqueous medium. This part can be related to a cone of semiangle θ, and thus the theoretical range of variation of the estimated distance, $r(\frac{2}{3})$, assuming $K_p^2 = \frac{2}{3}$ with respect to the real distance can be calculated [10]. Since this angle is highly dependent on the structural features of the protein bilayer complex, this factor has to be independently evaluated for each particular protein system.

3. Quantitation of the rate of energy transfer for protein systems undergoing lateral phase separation from the lipid, i.e., for eutectic lipid-protein mixtures

This kind of phenomenon can be treated as has already been done in the preceding paper [9] with phospholipid phase separation in the liquid crystalline state, but now using a donor group attached to the protein and an acceptor group attached to the lipid polar head group. Therefore, the condition of a much lower concentration of donor molecules than of acceptor molecules, established when deriving the formal equations for the rate of energy transfer in the preceding paper, can easily be satisfied. The formal equations are exactly the same except that protein parameters have to be used, i.e., protein monomer and phospholipid radii, and in this case the simplifications used in deriving $\bar{k}_i$ [9] clearly are much more justified because of the larger size of the protein. Moreover, an additional simplification of $\bar{k}_i$ can be introduced by neglecting all contributions to the rate of energy transfer except those coming from protein molecules of the outer shell of the protein cluster due to the large radius of the protein when compared to that of the phospholipid. Hence, eq. (28) of the preceding paper can be written for this case as

$$\langle k_T \rangle \approx 4 f_a k_1(\sigma)(\langle s \rangle - 1)/\langle i \rangle, \qquad (1)$$

where σ is now the sum of the protein monomer, σ_p, and phospholipid, σ_l, radii and the remaining symbols have the meanings defined previously [9] but are applied to protein instead of lipid clusters.

However, there is no need in this case to distinguish between gel and liquid lipid states in order to study the dependence of the average rate of energy transfer, since due to the perturbation of the lipid arrangements introduced by the protein and also due to the size ratio of protein to phospholipid molecules, the phospholipid space can be treated as a continuous medium.

4. Protein distribution on the vesicle surface: deviations from the random distribution

When aggregation and/or protein phase separation take place, the average distance between protein molecules decreases non-randomly with respect to the average distance in the case of random distribution of protein molecules on the vesicle surface. However, it must be distinguished theoretically and experimentally from protein aggregation and from eutectic mixture formation, since it is a physically different phenomenon that may have distinct biological consequences.

Unless the labelled acceptor phospholipid partitions out of the lipid-protein annulus, i.e., is partially or completely excluded from it and this case will be analyzed later in detail, eq. (17) of ref. [9] allows us to calculate that only the nearest two

shells of phospholipids contribute significantly, >90%, to the average rate of energy transfer between the protein and the surrounding phospholipids for a protein molecule of radius 25 Å. On the other hand, bibliographic data reveal that the lipid annulus of a protein molecule has, at least, one complete shell of phospholipid molecules [4,11–13] and that a minimum of two shells of phospholipid molecules will separate each pair of protein molecules. Therefore, to a first approximation, the phospholipid medium can be considered as isotropic with respect to the rate of energy transfer between donor proteins and acceptor phospholipid molecules for a non-random distribution of protein molecules, as opposed to what happens in the case of eutectic protein-lipid mixtures (see above) and alterations of the protein aggregation state (see below).

Donor-labelled protein and acceptor phospholipids also allow us to distinguish between random and non-random protein distribution if the labelled phospholid partitions out of the annular space surrounding the protein. Let us define the partition coefficient of the labelled phospholipid between the annular lipid space and the bulk lipid space, L, as follows:

$$L = (f_a)_{an}/(f_a)_b, \tag{2}$$

where $(f_a)_{an}$ and $(f_a)_b$ are the molar fractions of the labelled phospholipid in the annulus and in the bulk lipid, respectively. In this case, $k_1(r)$ can be expressed as the sum of two components (see appendix C and eq. (17) of the preceding paper),

$$k_1(r) = \tfrac{1}{16}\varepsilon\left[(f_a)_{an}(\sigma^{-4} - r_{an}^{-4}) + (f_a)_b(r_{an}^{-4} - r^{-4})\right], \tag{3}$$

where r_{an} is the radius of the protein annulus, $\varepsilon = B\pi/n_p$ and B the constant of the Förster equation: $k_T(r) = Br^{-6}$, giving the rate of energy transfer between a donor-acceptor pair at a distance r [14].

Combining eqs. (2) and (3) we obtain:

$$k_1(r) = (f_a)_b\left[k_1(\sigma) + \tfrac{1}{16}\varepsilon(L-1)(\sigma^{-4} - r_{an}^{-4})\right]. \tag{4}$$

Therefore, for a random distribution of protein monomers we can write

$$(\langle k_T\rangle)_R = (f_a)_b\left[k_1(\sigma) + (L-1)k(r_{an})\right], \tag{5}$$

where $k(r_{an})$ is given by the expression

$$k(r_{an}) = \tfrac{1}{16}\varepsilon(\sigma^{-4} - r_{an}^{-4}). \tag{6}$$

Let us consider a non-random distribution of protein molecules within the surface of a membrane as a phase separation of the protein-annular phospholipid system from the bulk lipid system. Since the phospholipid annulus (i) surrounding a protein molecule shows a structural organization different from that of the bulk lipid [4], it seems reasonable to assume that the excess energy requirements for the disruption of this lipid subphase will differentiate between protein phase separation if protein-protein interactions can overcome this energy barrier or merely a non-random distribution of protein molecules if they cannot. Bearing this in mind we can combine eqs. (1) and (5) and obtain:

$$(\langle k_T\rangle)_{NR} = (f_a)_b\left[4k_1(\sigma)(\langle s\rangle - 1)/\langle i\rangle + (L-1)k(r_{an})\right], \tag{7}$$

where all symbols have their usual meaning, but now $\langle s\rangle$ and $\langle i\rangle$ refer to the number of shells and to the number of protein-annular phospholipid, respectively, units in an average cluster.

Let us analyse now what the effect are of a non-random distribution of protein molecules on the average rate of energy transfer between two different batches of protein molecules, one labelled with the donor and the other with the acceptor. Since electrostatic forces are responsible for long-range interactions [15], it appears logical that, at least in some cases *, these forces will be responsible for a non-random distribution of protein molecules within the surface of the membrane. For the

* Although it has been shown [6] that protein phase separation can be qualitatively rationalized using the assumption of a demixing entropy of the lipid-protein system as the leading force, there is nothing, to the best of our knowledge, against the relevance of protein-protein interactions in this phenomenon. On the contrary, experimental data recently obtained in our laboratory using Ca^{2+}-ATPase from sarcoplasmic reticulum [16] strongly support this hypothesis.

simplest of these situations, the probability, $P(r)\,dr$, of finding a protein molecule at a distance between r and $r+dr$ from another one is

$$P(r)\,dr = 2\pi r \exp(-Q/rkT)\,dr. \quad (8)$$

where Q is a proportionality constant between the electric potential $\psi(r)$ and r^{-1}, i.e., $\psi(r) = Qr^{-1}$. Therefore, Q represents the net electric charge presented by a protein molecule to another. Note that the required differently charged regions within a protein molecule needed to justify such interactions can lead from a macroscopic point of view to very simple situations, such as that analysed above, due to preferred orientations among protein molecules, a fact that follows immediately from the initial assumption. Other electric contributions, such as dipole-dipole interactions, etc., can be neglected when compared to the electrostatic ones because of a stronger dependence on the intermolecular distance which will probably require that the partial or complete disruption of the annular lipid environment of protein molecules be significant and this seems to be unlikely in the case we are dealing with.

Using eq. (8) the value of the average distance in such a non-random distribution of protein molecules, $\langle r\rangle_{NR}$, within a spherical vesicle of radius R_g can be calculated by integrating over half of the sphere:

$$\langle r\rangle_{NR} = \frac{2\alpha'}{n_p - 1}\int_{\sigma_p}^{R_g} \pi r^2 \exp(-Q/rkT)\,dr, \quad (9)$$

where α' is a normalization factor such that

$$2\alpha'\int_{\sigma_p}^{R_g} \pi r \exp(-Q/rkT)\,dr = \tfrac{1}{2}(n_p - 1) \quad (10)$$

and n_p is the total number of protein molecules per vesicle.

Solving the integrals of eqs. (9) and (10) by Taylor's expansion of the exponential function, i.e., assuming that $Q/kT \ll 1$, and taking only the first two terms of this expansion we find

$$\alpha' = (n_p - 1)(4\pi)^{-1} \times \left[\tfrac{1}{2}\left(R_g^2 - \sigma_p^2\right) - (Q/kT)\left(R_g - \sigma_p\right)\right]^{-1} \quad (11)$$

and, therefore

$$\langle r\rangle_{NR} = \frac{\tfrac{1}{2}\left[\tfrac{1}{3}\left(R_g^3 - \sigma_p^3\right) - (Q/2kT)\left(R_g^2 - \sigma_p^2\right)\right]}{\left[\tfrac{1}{2}\left(R_g^2 - \sigma_p^2\right) - (Q/kT)\left(R_g - \sigma_p\right)\right]}. \quad (12)$$

On the other hand, for an ideal random distribution of protein molecules it can easily be shown that

$$\langle r\rangle_R = 2R_g/n_p^{1/2}. \quad (13)$$

Hence, for a totally random distribution the average distance between protein molecules depends on $n_p^{-1/2}$ whereas the average distance between protein molecules in the above-treated non-random distribution does not depend on n_p. Thus, using fluorescent derivatives of the protein molecule both situations can be distinguished.

5. Analysis of the geometric factors affecting the rate constant of energy transfer for several protein aggregation states

In the case of changes in the aggregation state of the protein, the phospholipid space around each protein monomer is perturbed from the first shell. Then, as discussed above, $\langle k_T\rangle$ should be affected greatly. To quantitate the effects of the aggregation state on the average $\langle k_T\rangle$ it is useful to assume that because of the relatively large radius of the protein, the contribution to $\langle k_T\rangle$ from the energy transfer between a labelled protein monomer and phospholipid molecules separated from it by at least one protein monomer can be neglected. Then, the value of $\langle k_T\rangle$ can be obtained using an area element that accounts for this effect instead of the area element, $dS = 2\pi r dr$, valid for a completely isotropic distribution of phospholipid around a protein molecule. Fig. 1 illustrates this effect. Quantitation of this can be done easily once γ is known (see fig. 1), considering the value of the central angle, α_0, that corresponds to the fraction of the space around each protein monomer, α, unaffected by the presence of the other subunits of the oligomer.

The calculated values of α for the most usual

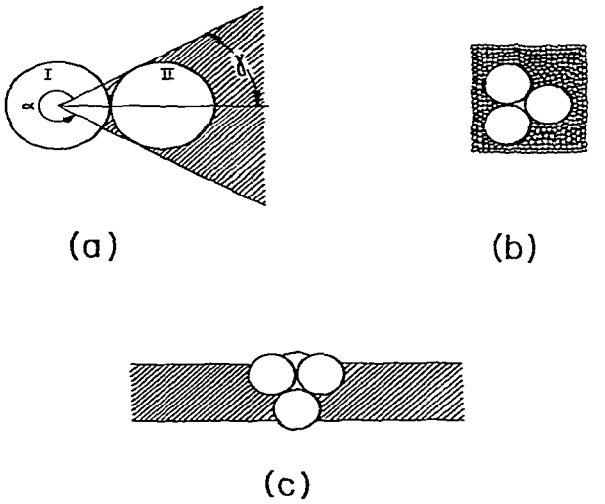

Fig. 1. (a) Schematic diagram showing the area of the phospholipid matrix around a protein subunit of a dimer (shaded area) perturbed by the presence of the other subunit. The relevant parameters α_0 and γ referred to in the text are shown in this figure. (b) and (c) are top- and cross-views, respectively, of the positions of the tetrahedral tetramer used to estimate the value of α for this aggregation state of the protein (see table 1).

aggregation states found in membrane proteins are given in table 1. For the sake of simplicity, in calculating these values it has been assumed that the value of γ (see fig. 1) for each monomer-monomer contact within an oligomer is always the same, i.e., that the oligomer is composed of identical subunits and that isologous intermonomer contacts are established [17].

Table 1

Fraction of the total protein surface, α, embedded in the lipid bilayer unperturbed by protein contacts within an oligomer

Aggregation state	Total surface directly exposed [b]	α
monomer	S_M [a]	1
dimer	$2S_M(\pi-\gamma)/\pi$	$1-\gamma/\pi$
trimer	$3S_M(2\pi-\pi/3-2\gamma)/2\pi$	$5/6-\gamma/\pi$
tetramer:	$4S_M(2\pi-\pi/2-2\gamma)/2\pi$	$3/4-\gamma/\pi$
–planar		
–tetrahedron	$\frac{1}{2}S_M(7/2-3\gamma/\pi)$	$(7/2-3\gamma/\pi)/8$
hexamer	$6S_M(2\pi-2\pi/3-2\gamma)/2\pi$	$2/3-\gamma/\pi$

[a] Surface of the protein monomer embedded in the bilayer.
[b] For simplicity, it has been assumed that the value of the central angle perturbation, γ, in each monomer-monomer contact within an oligomer is always the same. Each protein monomer has been assumed to be spherical and approximately equally immersed in the phospholipid bilayer.

Hence, we can write

$$(\langle k\rangle)_i = \alpha_i(\langle k_T\rangle)_{MR}. \tag{14}$$

where i refers to the aggregation state of the protein and $(\langle k_T\rangle)_{MR}$ is the average rate of energy transfer of a random distribution of protein monomers under the same experimental conditions, given by

$$(\langle k_T\rangle)_{MR} = f_a k_1(\sigma). \tag{15}$$

6. Quantitative dependence of the energy transfer efficiency on the protein concentration in the bilayer for simpler cases

A priori, we have two possible experimental approaches to study this problem depending on the chemical nature of the donor-acceptor pair used:

(i) Donor and acceptor are fluorescent derivatives of the protein.

(ii) Donor and acceptor are fluorescent derivatives of the protein and of the phospholipid, respectively.

It should be remarked that, as in the preceding paper [9], in both cases the number of acceptor molecules must be greater than that of donor molecules.

The most suitable approach is the latter for several reasons:

(a) Except for the case in which the labelled acceptor phospholipid partitions out of the protein annulus, there is no appreciable difference between the average rate of energy transfer for non-random and random distributions and then

$$(\langle \bar{k}_T\rangle)_{ONR}/(\langle \bar{k}_T\rangle)_{MNR} = (\langle \bar{k}_T\rangle)_{OR}/(\langle \bar{k}_T\rangle)_{MR},$$

where $(\langle \bar{k}_T\rangle)_{OR}$ and $(\langle \bar{k}_T\rangle)_{MR}$ are the average rate constants of energy transfer for a random distribution of oligomer and monomer molecules, respectively.

On the other hand, from eq. (14),

$$(\langle \bar{k}_T\rangle)_{OR}/(\langle \bar{k}_T\rangle)_{MR} = \alpha_i. \tag{16}$$

where α_i is the value of α for the oligomer (see table 1).

(b)If the distribution of monomers and/or oligomers is not entirely random, then the contri-

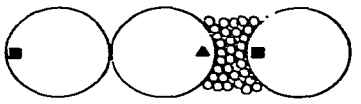

Fig. 2. Possible positions of the fluorescent labels, donor (▲) and acceptor (■), in a dimer and the closest distance between donor and acceptor groups located in different protein oligomers. Approximate protein monomer size in the diagram: $R_p = 25$–30 A. The probability of this situation is evidently enhanced either at low phospholipid-to-protein ratios, e.g., of the order of 100:1 (mol phospholipid per mol protein), or for proteins showing a non-random lateral distribution on the surface vesicle.

bution to the average rate of energy transfer arising from such a non-random distribution of protein molecules may eventually be very high if we are using donor and acceptor molecules attached to different protein molecules. Under these conditions it will be difficult, if not impossible, to calculate the contribution to the measured rate of energy transfer arising from oligomer (dimer, trimer, tetramer, etc.) formation. The situation is complicated even further by the fact that the distance between donor and acceptor fluorophores within an oligomer may eventually be similar to or even larger than the distance between monomers and/or oligomers in these non-random distributions of protein molecules. Fig. 2 illustrates this point of view for a simple case.

(c) Even if the distribution of monomer and dimer molecules is entirely random, at very high concentrations of protein in the bilayer, which is the case for some relevant proteins in natural membranes [1–3,18], the average distance between distinct molecules will be small and the complications mentioned in point (b) will appear. For example, a phospholipid/protein ratio of 60 gives an average of one shell of phospholipid around a protein monomer of molecular weight 10^5, randomly distributed. This implies an approximate average donor-acceptor distance of $2R_p + 20$ which can be compared to the maximum possible in a dimer of such a protein for which $R_p = 25$–30 Å, see fig. 2.

6.1. Monomer ⇌ dimer equilibrium involved

For this case, in general, can be written:

$$\langle \bar{k}_T \rangle = n_p^{-1} \left[(\langle \bar{k}_T \rangle)_{MNR} C_M + (\langle \bar{k} \rangle)_{DNR} C_D \right], \quad (17)$$

where C_M and C_D are the average concentrations of monomer and dimer in each vesicle, respectively, and $(\langle \bar{k}_T \rangle)_{MNR}$ and $(\langle \bar{k}_T \rangle)_{DNR}$ are the values of the average rate constants of energy transfer for the general case of non-random distributions of monomers and dimers.

Eq. (17) can also be written as

$$\langle \bar{k}_T \rangle = \left[(\langle \bar{k}_T \rangle)_{MNR} + (\langle \bar{k}_T \rangle)_{DNR} K C_M \right] \times C_M / n_p, \quad (18)$$

where $K = C_D / C_M^2$; n_p, the total number of protein molecules per vesicle and C_M is related to n_p and K by

$$C_M = \left[-1 + (1 + 8Kn_p)^{1/2} \right] / 4K. \quad (19)$$

Using fluorescent derivatives of the protein (donor) and phospholipid (acceptor), then bearing in mind that stated above and substituting eq. (16) in eq. (18) we obtain:

$$\langle \bar{k}_T \rangle = \left[1 + (1 - \gamma/\pi) 2KC_M \right] \times (C_M / n_p)(\langle \bar{k}_T \rangle)_{MR}. \quad (20)$$

Eqs. (19) and (20) allow determination of the monomer ⇌ dimer equilibrium constant K within a narrow range of uncertainty, provided that $\langle k_T \rangle_{MR}$ can be calculated using eq. (15) and that γ can be estimated within a narrow range for each protein system while only knowing its approximate structural features.

6.2. Monomer ⇌ dimer ⇌ tetramer equilibria involved

For this case and again using labelled protein (donor) and labelled phospholipid (acceptor):

$$\langle \bar{k}_T \rangle = n_p^{-1} \left[(\langle \bar{k}_T \rangle)_{MR} C_M + (\langle \bar{k}_T \rangle)_{DR} C_D + (\langle \bar{k}_T \rangle)_{TR} C_T \right], \quad (21)$$

where C_M, C_D and C_T are the concentrations of monomers, dimers and tetramers in each vesicle, respectively, and in $(\langle \bar{k}_T \rangle)_i$ (i = MR, DR and TR), the average rate of energy transfer of a random distribution of monomers, dimers and tetramers, respectively.

Defining $K_1 = C_D/C_M^2$ and $K_2 = C_T/C_D^2$ and considering eq. (16) and the values of α_i given in table 1 we can derive

$$\langle \bar{k}_T \rangle = \left[1 + 2(1-\gamma/\pi)K_1 C_M + \nu K_2 K_1^2 C_M^3\right] \times (C_M/n_p)(\langle \bar{k}_T \rangle)_{MR}, \quad (22)$$

where $\nu = (\langle \bar{k}_T \rangle)_{TR}/(\langle \bar{k}_T \rangle)_{MR}$.

Eq. (22) allows us to obtain the equation relating the average rate of energy transfer of this system to the association constants and the geometrical parameters γ and ν.

On the other hand, C_M is related to the association constants by the following equation:

$$C_M^4 + C_M^2/2K_1K_2 + C_M/4K_2K_1^2 - n_p/4K_2K_1^2 = 0 \quad (23)$$

(derived from $C_M + 2C_D + 4C_T = n_p$).

The parameters K_1, K_2 and ν can be obtained from the curve of $\langle \bar{k}_T \rangle$ versus n_p in the following way:

First, fitting that part of the curve corresponding to very low values of n_p, i.e., under conditions in which only the equilibrium monomer = dimer should be relevant. This analysis should allow us to obtain K_1 by fixing a value of γ (see the preceding section).

Second, fitting the whole curve by using the previously calculated K_1. Then, using each time a value of ν of those shown in table 1, one can determine the value of K_2 that gives the best fit. From the quality of the fit or from independently obtained structural data one then chooses the most suitable ν, if there is a preferred one, and then K_2.

Fig. 3 shows a plot of the rate of energy transfer as a function of the number of protein molecules per vesicle, n_p, for a self-associating protein system, and non-random and random protein distributions within the bilayer. It can be seen that self-association processes can easily be distinguished from a non-random distribution of the protein within the bilayer following the experimental approach outlined.

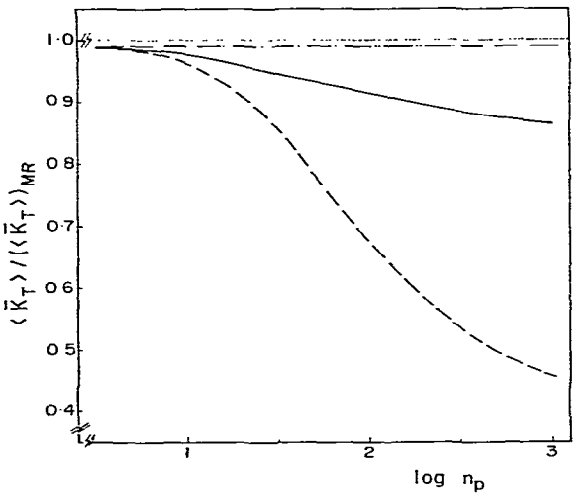

Fig. 3. Dependence of the relative average rate of energy transfer $\langle \bar{k}_T \rangle / \langle \bar{k}_T \rangle_{MR}$, between donors attached to the protein and acceptor phospholipid derivatives on the number of protein molecules per vesicle in the following cases: random (. . .) and non-random (– · · – · · –) distribution of protein molecules. A protein associating system has the relevant association equilibria: (a) monomer = dimer, $K = 0.01$ and $\gamma = \pi/6$ rad (————) and (b) monomer = dimer = tetramer, $K_1 = 0.01$, $K_2 = 0.1$, $\nu = \frac{1}{4}(\frac{7}{2} - 3\gamma/\pi)$ and $\gamma = \pi/6$ rad (— — —). The assumed fraction of each monomer surface merging with the water space for a semi-angle of $\approx \pi/8$. See the text for further details.

7. Concluding remarks

In this paper, the theoretical background has been developed for distinguishing between a random and non-random protein distribution in unilamellar vesicles, eutectic protein-lipid mixtures and aggregation processes of intrinsic membrane proteins from measurements of the rate of energy transfer among donor and acceptor molecules attached to protein and phospholipid components of the membrane. It should be emphasized that the experimental information needed to apply the equations reported here can be obtained under experimental conditions in which the protein systems are functionally active. In fact, the perturbation, introduced by attaching a fluorescent label to the protein, on its functional properties has been

shown to be negligible in several relevant membrane proteins [10].

Perhaps the most restrictive assumptions made in deriving most of the equations of this paper is the non-preferential binding of the labelled phospholipid to the protein or its exclusion from the lipid annulus. Therefore, the effect of the fluorescent label of the phospholipid on its binding to the protein must be tested prior to the start of this kind of study. To do this, complexes of the protein with the phospholipid (labelled and unlabelled) must be prepared using different ratios of labelled/unlabelled phospholipid, the total protein and phospholipid concentrations being constant in all cases. The average distance from the labelled phospholipid to the protein can be determined from experiment by using the equations derived above and the dependence of the calculated distance on the ratio of labelled/unlabelled phospholipid can be compared to that expected if no preferential binding or exclusion of the labelled lipid from the lipid annulus occurs. In doing so, an approximate value for the partition coefficient, L, of the phospholipid between the annulus and the bulk lipid can be determined and eqs. (1), (19) and (21) can easily be corrected to take into account this problem unless the phospholipid partitions out of the annulus and, hence, the latter situation has to be carefully avoided.

It should also be remarked that, because of the low protein molar fraction in the bilayer and also because of the low value of the fraction of the lipid labelled when doing energy transfer experiments, eventually the average energy transfer, $\langle E_T \rangle$, can provide the same information as does the average rate of energy transfer (see the preceding paper for a more detailed discussion [9]). Specifically, the whole discussion on this matter in the preceding paper is valid for the processes analyzed in sections 3 and 4 of this paper, i.e., for eutectic lipid-protein mixtures and for non-random protein distributions on vesicle surfaces but only the iterative method outlined there is valid for analysis of the protein aggregation state done in section 6 of this paper.

Finally, let us note that the information derived from these studies can be used to improve our knowledge of protein-lipid and protein-protein interactions in biological membranes and of the thermodynamics of the processes analyzed here.

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