

Use of Energy Transfer to Assay the Association of Proteins with Lipid Membranes

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A method is described for the rapid and quantitative assay of the association of proteins with lipid membranes. The method utilizes the nonradiative transfer of energy between fluorescent groups in the protein and fluorescent groups attached to the polar heads of the lipids in the membrane. The association constant for the electrostatic association of trypsin with bilayers of the negatively charged lipid, 1,2-dimyristoyl-*sn*-glycerol-3-phosphoric acid methyl ester (MPA) at a salt concentration of 5 mM and pH 8 was found to be $(2.32 \pm 0.07) \times 10^5 \text{ M}^{-1}$. The stoichiometry of lipid to protein in the association complex is 40–50 lipid molecules per trypsin molecule. Honey-bee venom melittin and the serum apolipoprotein, apo-Lp-Ala, were shown to associate with lecithin vesicles. Acetylcholine esterase isolated from the electric organ of the electric eel according to the method of Leuzinger and Baker [(1967) *Proc. Nat. Acad. Sci. USA* 57, 446–451] was shown to be incapable of associating with lecithin or MPA bilayers.

Little is known about the molecular interaction of proteins with lipids, which is basic for the function of biological membranes. As a first step in the study of lipid–protein interactions, a rapid and quantitative assay for the association of proteins with lipid membranes is required. We have developed such a method. It is based upon the nonradiative transfer of energy (1) between fluorescent chromophores in the protein and fluorescent groups attached to the head groups of the lipid in the membrane. The method allows observation of lipid–protein “association”, by which term we denote lipid–protein proximity of up to about 50 Å, and the assay of association parameters.

It follows from the theory of nonradiative energy transfer (1) that the efficiency of transfer is dependent, among other factors, upon (i) the separation distance between the donor and acceptor chromophores, and (ii) the extent of overlap between the emission spectrum of the donor and the absorption spectrum of the acceptor. Efficient transfer of energy in most donor–acceptor pairs occurs at separation distances of up to about 50 Å (2) and, since the radii of most proteins do not exceed this distance, their interaction with the lipid bilayer containing a suitable donor or acceptor chromophore can be observed.

Most proteins have intrinsic fluorescent groups such as the tyrosine and tryptophan residues, flavin and pyridoxal cofactors, etc., which may be used as either donors or acceptors. When the tyrosine or tryptophan residues are chosen as donors, a lipid acceptor having an absorption in the 300- to 380-nm range is required. DNS-kephalin is such a fluorescent lipid derivative. Energy transfer from the protein to the DNS group results in a sensitized fluorescence of the latter when the protein associates with the lipid.

As an illustration we have used the method to quantify the association between trypsin and 1,2-dimyristoyl-*sn*-glycerol-3-phosphoric acid methyl ester (MPA) at pH 8. We have also investigated the use of the method in detecting association of melittin, a serum apolipoprotein, and acetylcholine esterase from the electric eel with MPA and lecithin vesicles.

MATERIALS AND METHODS

DNS-kephalin (dimyristoyl) was synthesized as described by Waggoner and Stryer (3). MPA (dimyristoyl) was a gift from Dr. H. Eibl of this institute; lecithin (dipalmitoyl) was from Fluka AG, Buchs, Switzerland; 2 \times crystallized bovine pancreatic trypsin was from Worthington Biochemical Corporation, Freehold, New Jersey; melittin was from Serva Feinbiochemica, Heidelberg, West Germany; the serum apolipoprotein, apo-Lp-Ala, was a gift from Dr. V. W. Brown, La Jolla, California; and acetylcholine esterase, isolated from the electric organ of the electric eel according to the method of Leuzinger and Baker (4), was a gift from Dr. D. Nachmansohn, New York.

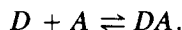
Lipid suspensions were prepared in 5 mM Tris-HCl, pH 8. The suspensions were sonicated for 3 min above the thermal phase transition temperature of the lipid, using a Branson Sonifier B-12 with a titanium microtip at 40 W. When lipid vesicles containing DNS-kephalin were prepared, the bulk lipid and DNS-kephalin in a molar ratio of 9:1 were mixed as chloroform solutions. The chloroform was then evaporated by blowing a stream of dry nitrogen over the solution and the residue was suspended in buffer at a temperature above the thermal phase transition temperature of the bulk lipid. Sonication of the resulting suspension was done at the same temperature for 3 min. Proteins were added to the lipid suspensions as concentrated solutions so that the final change in volume after addition of the protein was not more than 1%.

Fluorescence measurements were done on a Fica 55 absolute spectrofluorimeter at 25°C. Excitation and emission bandwidths were 7.5 nm. Cuvettes of 0.4-cm path length were used. Absorption spectra were obtained on a Cary 118 spectrophotometer at 25°C.

RESULTS AND DISCUSSION

The overlap of the fluorescence emission spectra of tryptophan and tyrosine and the absorption spectrum of the DNS chromophore is shown in Fig. 1. From its spectral characteristics, the DNS group is ideally suited to accept energy by the Förster mechanism from tryptophan and/or tyrosine as donor. In addition, the fluorescence emission spectrum of the DNS chromophore is well separated from the emission spectra of tryptophan and tyrosine, thus making it possible to observe energy transfer quite conveniently by following either the protein fluorescence or the sensitized acceptor (DNS) fluorescence. In this work we have used the latter, since sensitized acceptor fluorescence is both a necessary and a sufficient criterion for the demonstration of an interaction between the donor and acceptor.

In order to derive a phenomenological binding constant for the donor (e.g., protein)-acceptor (e.g., fluorescent lipid) association, two hypotheses have to be made in the simplest case: (i) Let the donor, D , the acceptor, A , and the association complex, DA , be related by a one-step reaction only:



The association constant is then

$$K_{DA} = [DA]/[D][A].$$

(ii) Let the concentration, $[DA]$, of the complex be proportional to the increase, $F_A([D]) - F_A(0)$, in the acceptor fluorescence intensity, F_A , due to the increase in the donor concentration, $[D]$. F_A is measured at 540 nm

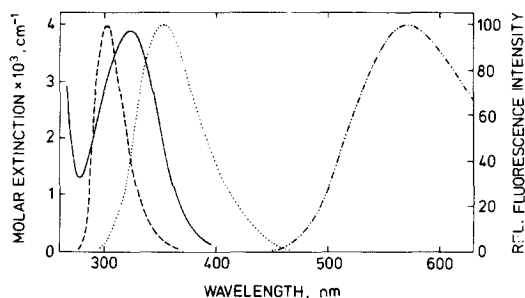


FIG. 1. Spectral overlaps of the fluorescence emission spectra of tyrosine and tryptophan with the absorption spectrum of the DNS chromophore. (—), Absorption spectrum of DNS-amide in water; (---), emission spectrum of N -acetyl-L-tyrosine amide in water; (·····), emission spectrum of N^{α} -acetyl-L-tryptophan amide in water; (- · - · -), emission spectrum of DNS-amide in water. All spectra were obtained at 25°C. Excitation and emission bandwidths for fluorescence spectra were 7.5 nm. Emission spectra are corrected and normalized.

for the use of DNS–kephalin as acceptor, and $[D]$ is known independently from the amount of protein added. For $[D] \rightarrow \infty$, $[DA]$ approaches the initial acceptor concentration, while F_A approaches asymptotically a maximum value, $F_A(\infty)$; therefore, the actual acceptor concentration, $[A]$, is equally proportional to $F_A(\infty) - F_A([D])$. The association constant is then given by

$$K_{DA} = \frac{1}{[D]} \frac{F_A([D]) - F_A(0)}{F_A(\infty) - F_A([D])} \quad [1]$$

$F_A(0)$, $F_A(\infty)$, and one intermediate measurement of $F_A([D])$ for a given protein concentration, $[D]$, are sufficient to calculate K_{DA} , but the hypotheses made are only consistent if all donor concentrations lead to the same phenomenological association constant.

As a demonstration of the validity of the method we have investigated the association of trypsin (isoionic point = pH 10.1) with MPA bilayers at pH 8 and a salt concentration of 5 mM. The interaction between the protein and lipid in this case is electrostatic in nature, i.e., coulombic attraction between the positively charged groups on the protein surface and the negatively charged polar head groups of the lipid molecules. Figure 2 shows the titration of trypsin association with MPA bilayers. The association between the protein and the lipid is manifested as the enhancement of DNS fluorescence upon excitation of the mixture at 280 nm. At this wavelength no discrimination is made between the excitation of tyrosine and tryptophan residues in the protein, and consequently both types of residues contribute to the energy transfer process. In Fig. 3 we have plotted the fluorescence intensity, F_A , of the DNS group at 540 nm in the presence of increasing concentrations of the protein. This figure also shows that the trypsin

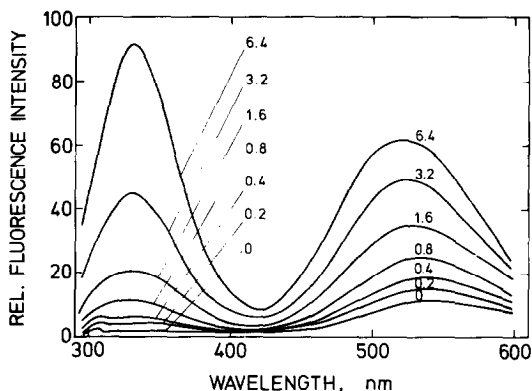


FIG. 2. Titration of a suspension of 10^{-4} M [MPA + DNS–kephalin (9:1)] in 5 mM Tris–HCl, pH 8, with increasing concentrations of trypsin. The final concentrations of trypsin (μ M) are indicated for each curve in the figure. In all cases the excitation wavelength was 280 nm. Excitation and emission bandwidths were 7.5 nm. Spectra are corrected.

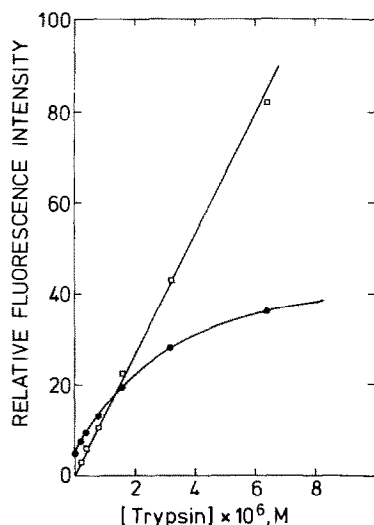


FIG. 3. Titration of MPA with trypsin. (●) F_A at 540 nm with 280-nm excitation in mixtures of 10^{-4} M [MPA + DNS-kephalin (9:1)] + trypsin in 5 mM Tris-HCl, pH 8, 25°C; (□) relative fluorescence emission quantum yield of trypsin fluorescence with 280-nm excitation in the presence of 10^{-4} M MPA in 5 mM Tris-HCl, pH 8, 25°C. In all cases excitation and emission bandwidths were 7.5 nm.

fluorescence is linear with concentration, in the absence of the DNS acceptor, in the range used by us in this work. The curve of DNS fluorescence versus trypsin concentration was extrapolated to infinite trypsin concentration on the basis of Eq. [1] derived above. From the value of $F_A(\infty)$ thus obtained, K_{DA} was calculated to be $(2.32 \pm 0.07) \times 10^5 \text{M}^{-1}$. We were also able to demonstrate that the association is somewhat stronger in the absence of salt and that trypsin does not interact with MPA bilayers in the presence of 0.5 M sodium chloride or with lecithin bilayer vesicles. The salt concentration dependence of trypsin association with MPA bilayers is a consequence of the dependence of the surface potential of the charged MPA bilayer upon the salt concentration (5). This result verifies the purely electrostatic nature of the trypsin-MPA interaction.

In order to investigate the stoichiometry of the trypsin-MPA association complex we titrated a fixed (1.25×10^{-6} M) concentration of trypsin in distilled water with MPA bilayer vesicles. The efficiency of the energy transfer process was then plotted versus the total lipid/protein molar ratio. The result is shown in Fig. 4. The intersection of the initial slope line and the asymptotic saturation value line gives the lipid/protein molar ratio in the complex. In the case under investigation this ratio was found to be between 40 and 50 lipid molecules per protein molecule. Trypsin is a globular protein with a molecular diameter of about 40 Å (6). The surface area per MPA molecule in a bilayer is about 45 Å² (unpublished X-ray diffraction

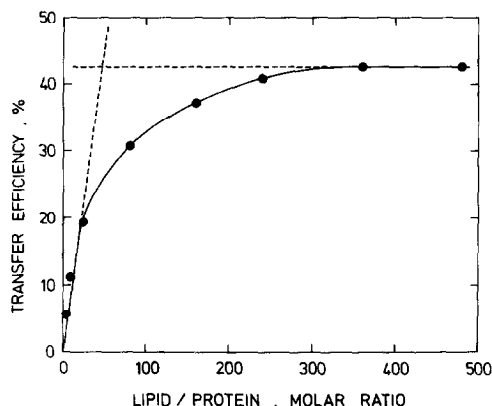


FIG. 4. Titration of a fixed (1.25×10^{-6} M) concentration of trypsin in distilled water with increasing concentrations of [MPA + DNS-kephalin (9:1)]. Transfer efficiency was calculated from the sensitized acceptor fluorescence.

results of K. Harlos). If the globular trypsin molecules are assumed to associate with the bilayer membrane only on the surface and without penetration of the membrane, then the observed stoichiometry has roughly twice as many lipid molecules per protein molecule than would be expected from simple area considerations. This could be due to one of two possible reasons. First, on association with the lipid the protein may partially unfold, but this is not likely since trypsin bound to MPA was observed to be catalytically active (H. Träuble, personal communication). The second and more likely possibility is that the MPA bilayers are in the form of closed vesicles which are not permeable to trypsin. Thus, the trypsin molecules are able to interact only with the outer surface of the bilayer vesicle.

The method has been used by us to qualitatively investigate the association of lipids with melittin (an amphiphilic polypeptide from honey-bee venom), apo-Lp-Ala (a serum apolipoprotein), and the acetylcholine esterase from the electric organ of the electric eel. As expected from other studies (7-9) melittin and apo-Lp-Ala do interact with lecithin bilayer vesicles. The titration of lecithin vesicles containing DNS-kephalin with increasing concentrations of the acetylcholine esterase is shown in Fig. 5. No association of the acetylcholine preparation used by us with either lecithin or MPA bilayers could be detected by us. The enzyme appears to be associated with the postsynaptic membrane in the natural state, at least as evident from cell-fractionation and histochemical techniques (10,11). It is possible that during purification of this enzyme by the method of Leuzinger and Baker (4) or upon storage of such a purified enzyme, a subunit or subunits which are essential for association with lipids, but not essential for enzymatic activity, are lost. While this manuscript was in preparation, Lwebuga-Mukasa *et al.* (12) have reported findings which confirm this view. Further

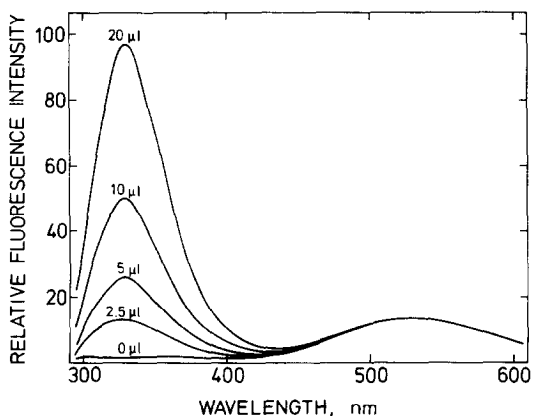


FIG. 5. Titration of 10^{-4} M [lecithin + DNS-kephalin (9:1)] with acetylcholine esterase in 5 mM Tris-HCl, pH 8.5, 25°C. Volumes indicated in the figures are the volumes of a solution of electric eel acetylcholine esterase ($OD_{280} = 4.1$, $OD_{254} = 2.9$) added to 1 ml of the lipid suspension. Excitation was at 280 nm. Excitation and emission bandwidths were 7.5 nm.

work done by us (K. Kaufmann and T. Rosenberry, in preparation) also confirms this opinion. In view of this, interpretation of results on membrane "reconstitution" with this protein must be treated with some caution.

It would be useful to consider some intrinsic limitations of the assay method described here. According to Förster theory (1) the efficiency of nonradiative energy transfer, E , between two chromophores separated from each other by a distance R is given by

$$1/E = 1 + (R^6/R_0^6),$$

where R_0 is the so-called critical transfer distance at which $E = 0.5$. In Fig. 6 we have plotted E versus R/R_0 . It is immediately seen that, due to the dependence of $1/E$ on R^6 , the efficiency of the transfer process becomes poor for $R > R_0$. Therefore, in general, in order for the assay described here to be useful the separation distance between the donor and acceptor chromophores should be equal to or less than R_0 . To some extent R can be manipulated by varying the DNS-kephalin ratio in the bulk lipid. For our work with trypsin, sufficiently efficient transfer of excitation energy was observed when the DNS-kephalin was as low as 5% of the total lipid. However, when varying the DNS-kephalin ratio it must be remembered that the DNS-kephalin molecule has a net negative charge and the influence of this charge on the electrical properties of the lipid membrane must be considered.

The critical transfer distance is defined as (1)

$$R_0 = (9.79 \times 10^3) (J_{AD} \kappa^2 \Phi_D n^{-4})^{1/6} \text{ \AA}.$$

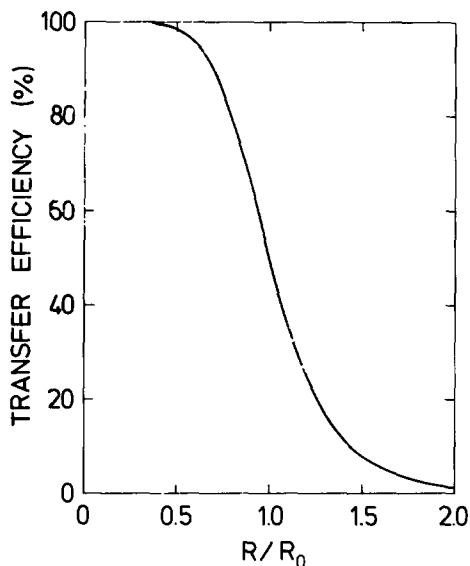


FIG. 6. The dependence of transfer efficiency upon the separation distance between the donor and acceptor chromophores as predicted by Förster theory (1).

Here J_{AD} is the spectral overlap integral for the donor-acceptor pair, κ^2 is the orientation factor, Φ_D is the donor fluorescence emission quantum yield in the absence of the acceptor, and n is the refractive index of the intervening medium. Ideally, for the purposes of this assay, R_0 should be large. In the work reported here we have used the Trp/Tyr-DNS pair for which the value of R_0 is about 20 Å (13). This may impose some limitations on the use of the assay for large proteins with a relatively low Trp and Tyr content. The acetylcholine esterase, with a molecular weight of about 250,000 and a total of four Trp and seven Tyr residues (4), is such a protein. However, it must be noted that in the case of this protein we were not able to observe any energy transfer even at very high protein concentrations.

The attractions of this assay include its rapidity, reproducibility, and the possibility of using the method to study rapid association processes.

Energy transfer has been used in the past to study protein-lipid interactions [for a review see Ref. (14)], but this is the first attempt at quantitating the association between proteins and lipids using this method. It should be of interest in experiments involving the development of "reversible" purification methods for membrane proteins, in "reconstitution" studies, and in the investigation of the lipid-association behavior of purified membrane proteins.

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