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A NEW FLUORIMETRIC METHOD TO MEASURE PROTEIN-CATALYZED PHOSPHOLIPID TRANSFER USING 1-ACYL-2-PARINAROYLPHOSPHATIDYLCHOLINE

P. SOMERHARJU [a], H. BROCKERHOFF [b] and K.W.A. WIRTZ [a]

[a] *The Biochemical Laboratory, Transitorium 3, Padualaan 8, 3584 CH Utrecht (The Netherlands) and* [b] *New York State Institute for Basic Research in Mental Retardation, 1050 Forest Hill Road, Staten Island, NY 10314 (U.S.A.)*

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A new, simple and versatile method to measure phospholipid transfer has been developed, based on the use of a fluorescent phospholipid derivative, 1-acyl-2-parinaroylphosphatidylcholine. Vesicles prepared of this phospholipid show a low level of fluorescence due to interactions between the fluorescent groups. When phospholipid transfer protein and vesicles consisting of non-labeled phosphatidylcholine are added the protein catalyzes an exchange of phosphatidylcholine between the labeled donor and non-labeled acceptor vesicles. The insertion of labeled phosphatidylcholine into the non-labeled vesicles is accompanied by an increase in fluorescence due to abolishment of self-quenching. The initial rate of fluorescence enhancement was found to be proportional to the amount of transfer protein added. This assay was applied to determine the effect of membrane phospholipid composition on the activity of the phosphatidylcholine-, phosphatidylinositol- and non-specific phospholipid transfer proteins. Using acceptor vesicles of egg phosphatidylcholine and various amounts of phosphatidic acid it was observed that the rate of phosphatidylcholine transfer was either stimulated, inhibited or unaffected by increased negative charge depending on the donor to acceptor ratio and the protein used. In another set of experiments acceptor vesicles were prepared of phosphatidylcholine analogues in which the ester bonds were replaced with ether bonds or carbon-carbon bonds. Assuming that only a strictly coupled exchange between phosphatidylcholine and analogues gives rise to the observed fluorescence increase, orders of substrate preference could be established for the phosphatidylcholine- and phosphatidylinositol transfer proteins.

Introduction

Since the discovery of the phospholipid transfer proteins a number of different methods to measure their activities have been described (for a review, see Ref. 1). These methods can be divided into two principal groups: the radiochemical procedures and spectroscopic techniques. In the former, transfer of a radioactive phospholipid from a donor to an acceptor membrane is measured, and typically the following steps are involved: (1) incubation of the donor and acceptor membranes in the presence of a transfer protein for a fixed time period; (2) separation of the two membranes by centrifugation or other means; (3) extraction of the acceptor lipids (not necessary in all procedures); (4) liquid scintillation counting of the lipid extracts and (5) calculation of transfer rates by using proper recovery corrections. Because of these many steps involved, the radiochemical methods are in general rather tedious and time consuming.

These drawbacks can be avoided by the use of spectroscopic techniques, which allow continuous monitoring of the reaction without separation of donor and acceptor membranes. If one neglects the NMR procedure described by Barsukov et al. [2], which requires prohibitively large amounts of lipids

to be generally acceptable, there is only one such method, that has been successfully used in studies of protein-catalyzed phospholipid transfer. This is the ESR method, originally described by Machida and Ohnishi [3]. This method makes use of a change in the ESR spectrum due to the transfer of spin-labeled phosphatidylcholine from labeled donor vesicles to non-labeled acceptor vesicles. The reaction can be continuously followed by recording the increase in the second derivative of the low field peak [4].

Here we describe a new spectroscopic method suitable for phospholipid transfer measurements. It is based on the observation that a large increase in fluorescence occurs when a fluorescent phospholipid is transferred from a membrane of fluorescent molecules into a membrane of non-labeled phospholipids. The fluorescent assay was applied to determine the effects of membrane charge and substrate modification on the transfer of phosphatidylcholine as catalyzed by the phosphatidylcholine-specific transfer protein from bovine liver [5], the phosphatidylinositol transfer protein from bovine brain, which transfers in addition to phosphatidylinositol also phosphatidylcholine [6] and the non-specific phospholipid transfer protein from rat liver which transfers all commonly occurring diacyl phospholipid species [7].

Materials and Methods

Lipids. Egg phosphatidylcholine was purchased from Makor Chemicals (Israel). Phosphatidic acid and lysophosphatidylcholine were prepared from egg phosphatidylcholine by phospholipase D or A_2 treatment, respectively [8,9]. Diether phosphatidylcholine (1-hexadecyl-2-(octadec-9-enyl)-*sn*-glycero-3-phosphocholine) and dialkylphosphatidylcholine (2-hexadecyl-2-(hexadec-9-enyl)-ethoxy-1-phosphocholine) were synthesized as described elsewhere [10]. All lipids gave a single spot on TLC using chloroform/methanol/concentrated ammonia/water (65 : 25 : 4 : 4, v/v) as solvent. *cis*-Parinaric acid was obtained from Molecular Probes (Plano, Texas) or was a gift from Professor R.D. Simoni. 1-Acyl-2-parinaroyl-*sn*-glycero-3-phosphocholine was synthesized from parinaroyl anhydride (190 μmol) and lysophosphatidylcholine (190 μmol) essentially as described by Gupta et al. [11]. At all stages of handling the parinaroyl lipids were kept under argon atmosphere and exposed only to dim green-yellow light to avoid oxidation and photochemical degradation. Parinaroylphosphatidylcholine was purified on a SiO_2 (Kieselgel 60, Merck) column using chloroform/methanol (65 : 35, v/v) saturated with argon as eluent. Elution of lipids was followed by analyzing each fraction by TLC and adsorption spectrometry. Fractions containing pure parinaroylphosphatidylcholine were pooled, taken to dryness and dissolved in 95% ethanol (Merck, fluorescence grade). 1 mol% butylated hydroxytoluene was added as antioxidant and the lipid was stored at −40°C in the dark. The yield was 67%. Assuming a molar absorption coefficient of 78 000 for the parinaroyl group in ethanol [12] the product was found to contain 0.97 mol parinaroyl residues per mol of phosphorus. This indicates that very little degradation or isomerization of the polyenoic fatty acid residue has taken place during the synthesis and purification. This was supported by the absorption spectrum, which was indistinguishable from the parinaric acid starting material. It should be noted that we found it necessary to use fresh, crystalline parinaric acid in this synthesis; with less pure preparates yields were poor.

Phospholipid transfer proteins. Phosphatidylcholine transfer protein from bovine liver and phosphatidylinositol transfer protein from bovine brain were purified as described earlier [5,6]. Non-specific transfer protein was purified from rat liver with a method to be published elsewhere.

Preparation of phospholipid vesicles. The donor vesicles consisting of the fluorescent phosphatidylcholine were prepared by the ethanol injection technique [13]. Sonicated acceptor vesicles were prepared as follows: The appropriate phospholipids were mixed with 1 mol% of butylated hydroxytoluene in chloroform, the solvent was evaporated and the residue was suspended in 20 mM Tris–HCl, 5 mM EDTA buffer, pH 7.4. The suspension was sonicated with a Branson B-12 sonifier at 10°C for 5–10 min under nitrogen and was then centrifuged at 27 000 × *g* for 15 min to remove titanium particles and any undispersed lipid. The recovery of the phospholipids in the supernatant was higher than 90%.

Fluorescence measurements. All fluorescence measurements were carried out at 25°C using a Perkin-Elmer MFP-3 fluorimeter equipped with a thermostated cuvette holder. The fluorimeter was operated

in the direct mode when the excitation spectra were recorded, while the ratio mode was used in the phospholipid transfer assay. Excitation and emission slits were 2 and 40 nm, respectively. To achieve continuous mixing during the assay measurements, a simple magnetic stirrer was attached to the cover of the cuvette compartment with the magnet closely above the cuvettes, which contained small magnetic bars. Without mixing, the fluorescence intensity was unstable, probably due to local bleaching of the label in the focused excitation beam. The assay buffer was filtered through a Millipore filter (0.6 μm) to remove dust particles.

Adsorption spectra were recorded with a Unicam (SP-1700) double beam spectrometer connected to a UNICOM AR 25 recorder.

Phosphorus was measured as described by Rouser et al. [14].

Results

Principle of the assay

Fig. 1 shows the excitation spectra of vesicles consisting of either pure 2-parinaroylphosphatidylcholine (trace A) or 2-parinaroylphosphatidylcholine premixed with a 30-fold excess of unlabeled phosphatidylcholine (trace B). The fluorescence of the parinaroyl groups is strongly quenched in the pure parinaroylphosphatidylcholine vesicles as compared to the vesicles in which the probe is diluted by non-labeled molecules. This quenching, which is obviously due to probe-probe interactions, provides the basis for a method to measure phospholipid transfer activities. When a phospholipid transfer protein is added to a mixture of pure parinaroylphosphatidylcholine vesicles and egg phosphatidylcholine vesicles, the protein will catalyze exchange of phospholipids between the labeled donor and non-labeled acceptor vesicles. This results in an increased fluorescence due to insertion of the labeled molecules into the non-labeled acceptor membrane.

Fig. 1 (trace C) shows the excitation spectrum when the phospholipid transfer protein has completely equilibrated the fluorescent probe between the donor vesicles and a 30-fold excess of the acceptor vesicles. The fluorescence intensity is about 55% of that observed for the vesicles of the premixed phospholipids (trace B). The difference in fluorescence intensity is due to the fact that only the outer monolayer phospholipids in the donor vesicles are available for protein-catalyzed transfer [15].

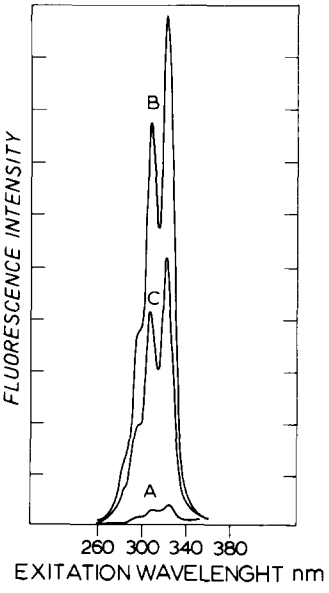

Fig. 1. Excitation spectra for pure parinaroylphosphatidylcholine vesicles (A), parinaroylphosphatidylcholine vesicles incubated for 30 min in the presence of vesicles containing non-labeled phosphatidylcholine and 1 μg of phosphatidylcholine transfer protein (C) and vesicles of premixed parinaroyl- and non-labeled phosphatidylcholine (B). Concentration of parinaroylphosphatidylcholine was 3.7 μM in each sample and that of non-labeled phosphatidylcholine (B and C) 100 μM. Emission wavelength 420 nm. Other conditions as given in Materials and Methods.

To determine phosphatidylcholine transfer activity the increase of the fluorescence intensity was measured at fixed excitation and emission wavelengths (324 nm and 420 nm, respectively). A typical assay procedure is carried out as follows (see Fig. 2): First the donor vesicles are prepared by injection of 2-parinaroylphosphatidylcholine in ethanol (5–10 μl) directly into the cuvette containing 2 ml of buffer. After a 10 min equilibration period the sonicated acceptor vesicles consisting of egg phosphatidylcholine are added (arrow B) and the background rate of fluorescence increase is recorded. Finally, the protein is added (arrow C) and the initial rate of fluorescence increase is recorded. This rate, after correction for the blank, was found to be proportional to the

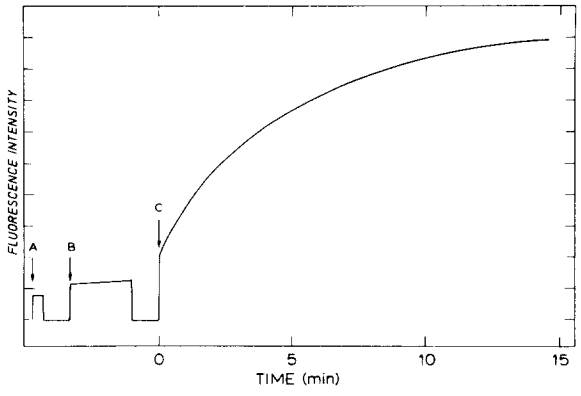

Fig. 2. Progress curve for protein catalyzed phosphatidylcholine transfer between labeled donor and unlabeled acceptor vesicles. Donor vesicles were prepared by injection of 10 nmol parinaroylphosphatidylcholine and 0.5 nmol phosphatidic acid dissolved in 10 μl ethanol into a cuvette containing 2 ml of 20 mM Tris-HCl, 5 mM EDTA buffer, pH 7.4 (arrow A). After 10 min of equilibration 200 nmol of sonicated egg phosphatidylcholine/phosphatidic acid acceptor vesicles (98 : 2 molar ratio) (arrow B) and 1 μg of phosphatidylcholine transfer protein (arrow C) were added. Excitation and emission wavelengths were 324 and 420 nm, respectively, and the chart speed 2.4 cm/min. Other conditions as given in Materials and Methods.

amount of protein added. No time-dependent increase in fluorescence was observed when heat denatured enzymes were used.

The background rate is usually low and seems to result from two different processes. One of these is very likely collision-dependent fusion and/or spontaneous exchange of phospholipids between the donor and acceptor vesicles. The other appears to be photochemical in nature since it is observed during irradiation of the donor versicles in the absence of acceptor vesicles.

Effect of membrane charge

Several previous studies have clearly shown that membrane charge has considerable effects on protein catalyzed phospholipid transfer in vitro. For instance, some groups have reported that a negative charge on the membrane inhibits the phosphatidylcholine transfer protein from bovine liver [3,16–18] while stimulation has been found by others [19]. The present spectroschopic assay has been used to obtain additional information on the effects of the negative charge of the membrane on the activity of this protein. Parinaroylphosphatidylcholine vesicles containing 3 mol% phosphatidic acid were used as the donor and sonicated egg phosphatidylcholine vesicles containing either 3 or 17 mol% phosphatidic acid as the acceptor. Initial rates of transfer as a function of acceptor concentration at a constant donor concentration (3.7 μM) are shown in Fig. 3A. At low acceptor concentrations (0–25 μM) initial rates were higher for the acceptor vesicles containing 17 mol% phosphatidic acid while at high concentrations (above 25 μM) the vesicles containing 3 mol% phosphatidic acid gave higher rates. It should be noted that the maximal rates were not affected markedly by the phosphatidic acid content of the acceptor versicles.

For comparison, we also determined the effect of membrane surface charge on the activity of the nonspecific phospholipid transfer protein from rat liver. In contrast to the phosphatidylcholine transfer protein this protein has a very broad substrate specificity and seems to be very sensitive to the ionic strength of the medium [20]. It is shown in Fig. 3B that the nonspecific protein behaved quite differently from the phosphatidylcholine transfer protein. Most importantly, the maximal rates were very much enhanced by increased negative charge of the acceptor vesicles. At high acceptor/donor phospholipid ratios initial

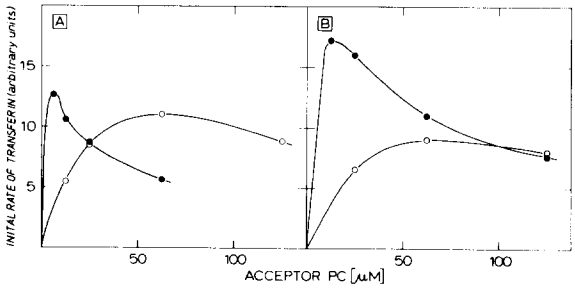

Fig. 3. Effect of acceptor membrane charge on transfer of phosphatidylcholine by the phosphatidylcholine (A) and non-specific (B) transfer proteins. The donor vesicles were prepared by injecting 7 nmol parinaroylphosphatidylcholine (containing 3 mol% phosphatidic acid) into 2 ml of Tris-EDTA buffer. The acceptor vesicles consisted of unlabeled phosphatidylcholine and either 3 (○) of 17 (●) mol% phosphatidic acid. Reactions were initiated by the addition of 0.2 μg of phosphatidylcholine transfer protein or 2.5 μg of nonspecific transfer protein. Conditions otherwise as in Fig. 2. These experiments have been repeated several times with virtually identical results.

rates were comparable for both acceptor versicles showing no marked inhibition by negative charge.

Effect of acceptor phosphatidylcholine modification

We also used the fluorimetric assay system to test the substrate specificities of phospholipid transfer proteins. It was assumed that under the conditions used, the proteins would catalyze a true exchange reaction, i.e; transfer of a phospholipid molecule from one type of membrane to the other is counterbalanced by an equimolar transfer of molecules in the opposite direction. Thus, if modification of the substrate would impair the complex formation with the transfer proteins, membranes consisting of this modified substrate could not participate efficiently in the exchange cycle, and consequently, lower rate of fluorescence increase should be observed when these membranes are used as acceptors. The following phosphatidylcholine analogues were used: (1) the normal diester (egg) phosphatidylcholine; (2) a diether analogue, which contains ether linkages between the glycerol residue and the hydrocarbon chains and (3) a dialkyl analogue, where the alkyl chains are connected to the 'glycerol' backbone via carbon-carbon bonds (for exact structures, see Materials and Methods). All three lipids have similar effective hydrophobic chain lengths and degree of unsaturation. Sonicated vesicles consisting of one of these analogues and 2 mol% phosphatidic acid were mixed with a constant amount of parinaroylphosphatidylcholine donor vesicles, and after addition of the transfer protein, the initial rate of fluorescence increase was recorded.

Fig. 4 shows the results obtained for the phosphatidylcholine transfer protein from bovine liver (A), the phosphatidylinositol transfer protein from bovine brain (B) and the non-specific phospholipid transfer protein from rat liver (C). For the phosphatidylcholine transfer protein highest rates were obtained with acceptor vesicles of the diester analogue. A 20–25% reduction in the rate of transfer was observed with the diether analogue while the dialkyl analogue was hardly transferred. Thus, this protein seems to be rather sensitive towards modifications in the hydrocarbon-polar head group region of the phosphatidylcholine molecule. A quite different behaviour was displayed by the phosphatidylinositol transfer protein (Fig. 4B). The rates obtained were practically independent of the phosphatidylcholine analogue used as the acceptor lipid. This suggests that the modified region does not play an important role in the stabilization of the complex between lipid and phosphatidylinositol transfer protein. Also the non-specific transfer protein seems to be less discriminative, since similar rates were obtained for the dister and diaklyl analogues. However, reduced rates were observed for the diether species (fig. 4C).

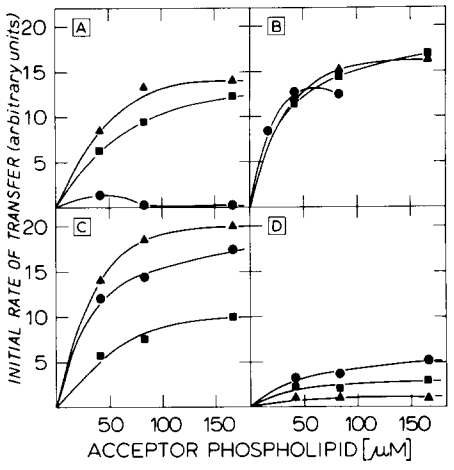

Fig. 4. Effect of phosphatidylcholine modification on the transfer activity of phosphatidylcholine (A), phosphatidylinositol (B) and non-specific transfer proteins (C). The donor vesicles consisted of parinaroylphosphatidylcholine (7 nmol) and phosphatidic acid (0.7 nmol), and the acceptor vesicles of 2 mol% of phosphatidic acid and 98 mol% of either diester (▲), diether (■) or dialkyl (●) phosphatidylcholine. 0.4 μg of phosphatidylcholine transfer protein, 1.8 μg of phosphatidylinositol transfer protein and 5 μg of non-specific transfer protein were used. Other conditions as in Fig. 2. (D) Rates in the absence of protein.

The background rates (Fig. 4D) were highest for the dialkyl and lowest for the diester analogue, the diether one giving intermediate values. These differences imply that the modification of the phosphatidylcholine molecules influences the stability of the corresponding vesicles towards fusion and/or spontaneous exchange of phospholipids.

A direct comparison of the rates obtained with different acceptors is feasible only if the fluorescence of the labeled lipid is the same in each acceptor membrane. To establish this, vesicles were prepared of 5 nmol of parinaroylphosphatidylcholine and a

40-fold molar excess of phosphatidylcholine analogue. The fluorescence intensity of the vesicles was found to be independent (less than 10% difference) of the phosphatidylcholine analogue used.

From the data in Figs. 4A–C we have calculated the relative rates of phosphatidylcholine transfer for the three different transfer proteins (arbitrary units per mol protein). If the rate of transfer is set at 1.0 for the phosphatidylcholine protein, the corresponding values for the phosphatidylinositol and non-specific phospholipid transfer protein are 0.38 and 0.07, respectively.

Discussion

Parinaric acid is a natural fatty acid which has been established as a most versatile fluorescent probe of lipid-lipid and lipid-protein interactions by Sklar, Simoni and associates [21–23]. In this study we present a fluorimetric assay for phosphatidylcholine transferring proteins based on the use of parinaroyl phosphatidylcholine. This fluorimetric method offers several advantages over the radiochemical procedures. (1) It is simpler and faster, since separation of acceptor and donor vesicles, lipid extraction and recovery calculations are not required. In addition, blank rates are conveniently obtained from the sample cuvette before addition of the transfer protein. (2) True initial rates are obtained for a wide range of enzyme concentrations, which is a useful feature, especially when samples of unknown activity (column fractions, for instance) are measured. (3) This method is very suitable for kinetic experiments; for example, the values of K_m and V can be obtained from a single progress curve [24]. (4) Both the donor and acceptor membrane are devoid of any undesirable biological activities and can be modified at will.

Our present studies demonstrate that an increase in the negative charge of the membrane can either stimulate or inhibit facilitated phospholipid transfer or have no effect depending on the protein and acceptor/donor ratios used. This is in agreement with the suggestion of DiCorleto et al. [19]. The phosphatidylcholine transfer protein from bovine liver was apparently stimulated by the negative charge at low acceptor concentrations, while inhibition was observed at higher concentrations. This result can be understood by taking into account the facts that this protein acts as a freely diffusing phospholipid carrier, and that it has higher affinity for the more negatively charged membranes [18,25,26]. At low acceptor concentrations the additional negative charge will enhance binding and consequently, the probability of a productive interaction between the protein and the acceptor membrane, which in turn results in increased rate of exchange. At high acceptor concentrations (i.e. high acceptor/donor ratios) the increased affinity for the acceptor vesicles will impair exchange of phospholipids between the donor and acceptor because of the decreased probability for protein-donor interaction. It is interesting to note that the non-specific transfer protein from rat liver responds quite differently to an increase in negative charge. Maximal rates of transfer were much higher for the more negatively charged acceptor vesicles. In addition, stimulation of the transfer by the negative charge was abolished at an acceptor/donor ratio which was 5-fold higher than that for the phosphatidylcholine transfer protein (see Fig. 3). It requires further investigations to establish whether this different behaviour is due to differences in mode of action.

In studies on phospholipid transfer substrate specificity is a question of major importance. Traditionally this problem has been approached by following transfer of radioactive lipids from a monolayer to vesicles [6] or between two separable vesicle populations [20,27]. Although these methods are usually reliable, they require radiolabeled substrates, which are not always easily available. To test an alternative approach, we studied how modifications in the structure of acceptor phosphatidylcholine would affect the rates of transfer of parinaroylphosphatidylcholine to vesicles consisting of these modified lipids. Assuming that only a strictly coupled exchange of lipids takes place between the donor and acceptor membranes, and that the formation of the lipid-protein complex is the rate limiting step in the exchange process, any modification that affects the complex formation should also affect the rate of parinaroylphosphatidylcholine transfer. It has been demonstrated that the phosphatidylcholine transfer protein from bovine liver catalyzes 1 : 1 exchange of phosphatidylcholine between two vesicle populations consisting of either the same or different molecular species [28]. Although less rigorously shown, it is likely that the phosphatidylinositol transfer protein will also cata-

lyze a 1 : 1 exchange of phosphatidylcholine since its mode of action seems to be very similar to that of the phosphatidylcholine transfer protein [29].

The present study indicates that there are considerable differences in phosphatidylcholine binding between the phosphatidylcholine and phosphatidylinositol transfer proteins. The phosphatidylcholine transfer protein shows the order of preference: diester, diether and dialkylphosphatidylcholine (Fig. 4A). Thus, an increase of the number of oxygen atoms in the region linking the polar and non-polar parts of the molecules seems to result in enhanced stability of the protein-lipid complex. It should be noted that the phosphatidylcholine analogues have, in addition to identical head groups, similar hydrocarbon chains, and are virtually isoteric. It would seem, therefore, that the preference of the protein for carboxyl ester groups indicates that in at least some of the lipid-protein complexes there is a requirements for hydrogen bonding in addition to electrostatic and hydrophobic bonding forces [30]. On the other hand, the phosphatidylinositol transfer protein can exchange phospholipids between the donor and acceptor at a rate which is practically independent of the type of acceptor lipid (Fig. 4B). This suggests that the modified region is less important in the stabilization of the phosphatidylcholine-phosphatidylinositiol transfer protein complex. In agreement with its low specificity, the non-specific transfer protein gave similar rates with the diester and dialkyl substrates. For some reason, however, lower rates were recorded for the diether analogue (Fig. 4C). Interpretation of these results in terms of substrate specificity is complicated by the fact that the mode of action of this protein is still largely unknown. It has been shown that this protein is able to catalyze net transfer of phospholipids from vesicles to delipidated lipoprotein and rat liver inner mitochondrial membranes [31]. Thus, it is uncertain whether exchange of phospholipid between the donor and acceptor vesicles is the only process contributing to the fluorescence increase when this protein is used. In conclusion we believe that the approach described here will be useful in studies on the substrate interactions of phospholipid transfer proteins, especially when comparative experiments are carried out with several purified proteins of different specificity.

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