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Interaction of clathrin coat proteins with unilamellar and multilamellar vesicles of phosphatidylcholine

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The binding of clathrin and accessory coat proteins to small unilamellar vesicles and to liposomes of uncharged phospholipids has been followed by chromatography, ^{31}P-NMR, ESR and fluorescence anisotropy. At pH 6.5 and at an ionic strength value (0.1 M Mes) close to that used during the purification of clathrin-coated vesicles, the proteins do not restore the characteristic network found around the natural vesicles. Instead, a limited fusion leads to enlarged structures in which the perturbation of the dynamics of the phospholipids decreases gradually with the depth in the membrane. While the rate of motion of the outer polar heads is lowered, the order parameter of doxyl groups located either under or in the vicinity of the glycerol backbone is not affected by the proteins. In the inner core of the membrane, the main thermotropic transition of the hydrocarbon chains is unchanged. All the effects are the results of interactions limited to the membrane surface. The electrostatic nature of these interactions is evidenced when the embedded spin labels have a charge protruding at the membrane surface. An 'anchoring' effect appears which is due to the charged groups of the proteins. The lateral diffusion of the probes is reduced and, at low ionic strength, a cationic derivative no longer detects the thermotropic transition of the hydrocarbon chains. These results indicate that, although it is known that clathrin and accessory proteins bind to membranes by a series of protein–protein interactions, this system is not devoid of lipid–protein interactions, at least when it is not organized as in the natural system.

Introduction

Receptor-mediated endocytosis of specific molecules by animal cells occurs via clathrin coated pits [1,2]. Despite some controversy [3], it is generally accepted that the complete budding of the pits leads to a scission and to individualized membrane bilayers enclosed by a polyhedral protein lattice. The major component of this network is a triskelion-shaped unit formed by three heavy chains and three light chains of clathrin [4,5]. The structure is completed by accessory proteins of 50, 100 and 115 kDa [6,7]. According to the model proposed by Vigers et al. [8], all the protein components are organized in three successive shells around the phospholipid bilayer. The inner shell is comprised of the accessory proteins. The intermediate shell corresponds to the terminal domain of clathrin heavy chains, while the outermost one corresponds to the other parts of the clathrin molecule.

Clathrin-coated vesicles are, thus, highly organized multimolecular complexes stabilized by protein–protein and protein–lipid interactions. Inside these complexes, all the experiments which will give a complete and precise picture of the different constraints imposed by elements to others will reveal the relative importance of the two types of interaction and will contribute to the understanding of the mechanism leading to the coated pit formation.

The present work reports on the interaction of clathrin and accessory proteins with unilamellar and multilamellar vesicles of phosphatidylcholine at pH 6.5. By

Abbreviations: Mes, 4-morpholineethanesulfonic acid; Tris, 2-amino-2-hydroxymethylpropane-1,3-diol; EDTA, ethylenediaminetetraacetic acid; EGTA, ethyleneglycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; DTT, dithiothreitol; PMSF, phenylmethylsulfonyl fluoride; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; DMPC, L-α-dimyristoylglycerophosphocholine; 5-AS, 5-doxylstearic acid; DPLNO, 5-doxyldipalmitoylphosphatidylcholine; CAT 16, 4-(*N,N*-dimethyl-*N*-hexadecyl)ammonium-2,2,6,6-tetramethylpiperidine-1-oxyl, iodide; egg PC, egg yolk phosphatidylcholine; DPH, diphenylhexatriene; DOPC, dioleoylphosphatidylcholine; DPPC, dipalmitoylphosphatidylcholine.

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analyzing the fluorescence and ESR signals of probes included in the bilayers, we show that perturbations can be induced which are not limited to the fusion of the vesicles. This indicates that these extrinsic proteins can interact with the phospholipids, at least when the system is not as well organized as the natural one.

Materials and Methods

Coated vesicles purification. Coated vesicles were purified according to a procedure derived from Nandi et al. [9]. Four bovine brains were stored on ice and treated 1 h after the death of the animals in the slaughterhouse. After removal of the meninges and cerebellum, the tissues were diluted with an equal volume of buffer (0.1 M Mes, pH 6.5, 1 mM EGTA, 0.5 mM $MgCl_2$, 0.02% NaN_3, 0.005% PMSF, 0.1 mM DTT).

Following Pearse [10], the tissues were then homogenized in a blender for three 10-s intervals. The homogenate was centrifuged for 30 min at $23500 \times g$ (12000 rpm in a Sorvall GSA rotor) in order to remove the solid material. The supernatant was then centrifuged for 90 min at $106000 \times g$ (30000 rpm in a Beckman rotor 30). The pellets of crude coated vesicles were resuspended in the extraction buffer, homogenized with a Dounce homogenizer and centrifuged for 10 min at $12000 \times g$ (10000 rpm in a Sorvall SS34 rotor). The last two high- and low-speed centrifugations were repeated before starting a new centrifugation in a solution of sucrose. Four fractions of 19 ml of crude coated vesicles were carefully layered on four centrifugation tubes each containing 20 ml of 8% sucrose/90% ^2H_2O (0.1 M Mes, 1 mM EGTA, 0.5 mM $MgCl_2$, 0.02% NaN_3, 0.005% PMSF, 0.1 mM DTT; pH measured, 6.3). The tubes were centrifuged for 130 min at $131000 \times g$ (27000 rpm in a Beckman SW27 rotor; temperature, 20°C). Pellets were then washed with the extraction buffer, diluted, homogenized and centrifuged again at $106000 \times g$ in order to remove traces of sucrose. The resultant pellets of purified coated vesicles were resuspended in the extraction buffer and stored at 4°C.

Considering the tendency of clathrin-coated vesicles to aggregate, samples were subjected to low-speed centrifugation before starting any further experiment.

When needed, coated vesicles were further purified by chromatography on a Sephacryl S-1000 gel (Pharmacia). Coated vesicles (5–7 ml, 2 mg/ml) were loaded onto a column (60 × 2 cm) which was equilibrated in the extraction buffer and presaturated with small unilamellar vesicles of egg PC. The elution profile contained two peaks. Purified coated vesicles were collected by pooling fractions of the first half of the second peak and characterized by 7.5% polyacrylamide disc gel electrophoresis and electron microscopy [11].

Purification of clathrin coat proteins. Clathrin coat proteins were extracted from clathrin-coated vesicles by dialyzing for one night against 1 M Tris (pH 7.3), 1 mM EDTA and 0.1% β-mercaptoethanol. Stripped vesicles were centrifuged for 50 min at $100000 \times g$. It was ascertained, by SDS-polyacrylamide gel electrophoresis, that under these conditions and according to Wiedenmann et al. [12], the supernatant contains mostly heavy and light chains and significant amounts of polypeptides of 100 and 50 kDa.

Preparation of clathrin empty coats. Clathrin coat proteins in 1 M Tris (pH 7.3), 1 mM EDTA and 0.1% β-mercaptoethanol were allowed to polymerize by overnight dialysis against a large volume of 0.1 M Mes buffer (pH 6.5). Aggregates were eliminated by low-speed centrifugation before loading on a Sephacryl S-1000 gel filtration column. Polymerized structures were analyzed by electron microscopy. Negative staining demonstrated that nearly all the particles examined contained the characteristic polygonal network of clathrin.

Protein-lipid complexes. DMPC was purchased from Sigma and egg PC was prepared and purified in the laboratory according to Singleton et al. [13]. Lipids were shown to be pure by thin-layer chromatography on silica gel plates developed in $CHCl_3/CH_3OH/NH_4$ (65:25:5, v/v) and $CHCl_3/CH_3OH/CH_3COOH/H_2O$ (75:45:12:6, v/v). Multi- and bilayer vesicles were prepared from lipid solutions in chloroform containing the appropriate concentration of spin-label or fluorescence probe. The film obtained after complete solvent evaporation was hydrated with 1 M Tris (pH 7.3), 1 mM EDTA and 0.1% β-mercaptoethanol. Liposomes were obtained by hand-shaking above the transition temperature of lipids. Small unilamellar vesicles were prepared by sonication at temperatures above 24°C for 5 min using an instrument manufactured by OSI, France, at an amplitude of 6 μm peak-to-peak and a frequency of 21 kilocycles/s. Under these conditions, 1 min was sufficient to reduce the light scattering of the lipid solutions. Subsequently, the preparations were centrifuged either at $145000 \times g$ for 1 h or at $27000 \times g$ for 30 min and stored at 32°C.

The complexes were made from stock solutions of coat proteins at concentrations of around 4 mg/ml by adding enough lipid liposomes or vesicles to give the desired protein/lipid ratio. The samples were then dialyzed against 0.1 M Mes (pH 6.5), 1 mM EGTA, 0.5 mM $MgCl_2$ and stored at 32°C.

Electron microscopy. Electron microscopy observations were performed on samples applied to copper grids coated with a film of formvar. Clathrin coats were negatively stained for 1 min with a buffered solution of 1% ammonium molybdate, dried by blotting with filter paper and examined in a Siemens Elmiscop 102 electron microscope operating at 60 or 80 kV. Size determinations were made on micrographs of random fields printed at a final magnification of 100000×. Possible

distortions due to partial flattening or non-uniform staining were corrected by considering the diameter of individual particles as the mean of measurements taken in two orthogonal directions.

ESR measurements. Spectra were recorded at 9.3 GHz on a Bruker ER200D spectrometer equipped with a field frequency lock and a temperature control unit. The temperature of the sample was measured inside the cell with a copper-constantan thermocouple. The sweep field was 100 G at 100 kHz modulation frequency and 1.0 G modulation amplitude. When needed, spectra were accumulated in a Bruker BNC12 computer. Results are reported using the outer hyperfine splitting $A_{\parallel}$ as a measure of the amplitude of motion of the fatty acid chain in the lipid bilayer. 5-AS and CAT 16 were purchased from Molecular Probes.

Fluorescence measurements. Static fluorescence measurements were performed on a Kontron SFM25 instrument interfaced to an Apple IIe computer. Temperature was regulated to ±0.2°C by means of a refrigerated water bath. Fluorescence anisotropies were measured by using excitation and emission polarizers according to the method of Azumi and McGlynn [13]. In this mode, the instrument was automated to measure fluorescence intensities sequentially and repetitively in all polarizer positions and to calculate anisotropies and standard errors.

^{31}P-NMR measurements. ^{31}P-NMR spectra (proton decoupled) were run at 121 MHz on a Bruker AM 300 WB spectrometer operating in the Fourier transform mode. All samples were diluted with 20% $^{2}H_2O$ for field stabilization.

Spin-lattice relaxation times (T_1) were obtained by the inversion recovery method. The amplitude of normalized spectra recorded at ten different delays was analyzed and fitted to an analytical function by the non-linear least squares method.

Results

Characterization by Sephacryl S-1000 chromatography

The interaction between clathrin coat proteins and large PC small unilamellar vesicles was first characterized by Sephacryl S-1000 gel chromatography (Fig. 1) (lipid

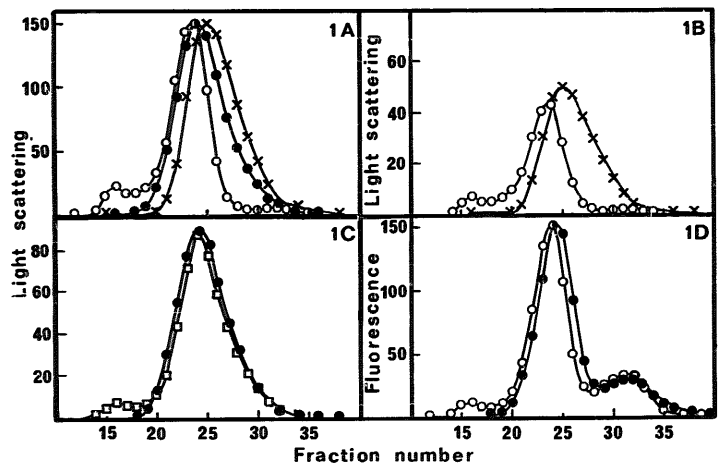

Fig. 1. Binding of clathrin coat proteins to phospholipid bilayers evidenced by chromatography on Sephacryl S-1000. (A) Elution profile detected by light scattering at 436 nm (signals normalized at the height of the peak). ×, freshly sonicated small unilamellar vesicles of phosphatidylcholine (2 ml were loaded on a column, 45×1.2 cm; flow rate, 3.8 ml/h); ○, reassembled clathrin coat proteins (triskelion concentration $1.9 \cdot 10^{-6}$ M); ●, reconstituted protein-lipid complex (molar ratio, $4 \cdot 10^{-4}$; lipid concentration, $5 \cdot 10^{-3}$ M). (B) Elution profile detected by light scattering. Non-normalized signals. ×, vesicles of phosphatidylcholine; ○, reassembled clathrin coat proteins. (C) Comparison of a computed signal expected from a hypothetical mixture of unaffected coats and vesicles (□) and the experimental signal (●). (D) Elution profile detected by the fluorescence emission of tryptophan (λ_{ex} 280 nm; λ_{em} 350 nm). ○, reassembled clathrin coat proteins; ●, reconstituted protein-lipid complex. Solvent: 0.1 M Mes (pH 6.5), 0.5 mM $MgCl_2$, 1 mM EDTA and 0.02% NaN_3.

concentration, $5 \cdot 10^{-3}$ M; triskelion/lipid ratio, $4 \cdot 10^{-4}$; 100 mM Mes, pH 6.5). When detected by light scattering, the elution profile exhibited only one peak which was compared to those obtained successively with pure proteins and lipids.

In the absence of lipids, when the proteins (triskelions and associated proteins) are dialyzed against 0.1 M Mes (pH 6.5), characteristic clathrin empty coats are formed. In electron microscopy and before gel filtration, these particles give rise to a bimodal size distribution centered on 640 and 740 Å [11]. On Sephacryl S-1000, the elution profile detected by light scattering (Fig. 1A) exhibited three peaks. Each of them contained fluorescent proteins as evidenced by the fluorescence emission of tryptophan (Fig. 1D). The first one (at the void volume of the column) corresponds to aggregates, the second one to the coats obtained on electron microscopy and the third one to a fraction of a lower hydrodynamic volume which may contain a minor component of high fluorescence quantum yield or a part of clathrin unable to polymerize after either partial denaturation or complete exhaustion of assembly proteins.

In the absence of proteins the expected diameter of pure egg PC unilamellar vesicles freshly sonicated and dialyzed as the two previous samples is about 240 Å [15]. That is the reason why this sample elutes later as illustrated either by normalized (Fig. 1A) or non-normalized signals (Fig. 1B).

When the proteins were added to the lipids, the formation of a lipid-protein complex was ascertained in the following way. First, Fig. 1C shows that the new experimental elution profile was different from that of a simple hypothetical mixture of pure unmodified vesicles and reassembled pure protein coats. Secondly, in the presence of proteins, the fusion of the small unilamellar vesicles occurs (see also the ^{31}P-NMR and the ESR experiments reported below and the results reported by Hong et al. [16]). Rather homogeneous structures were formed of diameter between 210 and 700 Å. Third, the fluorescence signals indicated that clathrin coat proteins follow these structures, i.e., a stable complex was formed (in good agreement with previous works [17–19]).

Electron microscopy

It was verified that in the presence of lipids, the proteins did not reassemble into coat structures around the vesicles at a low protein/lipid ratio. On the contrary, Fig. 2 shows that when limited amounts of protein are added to the phospholipids in 10 mM Mes (triskelion/lipid ratio $2.5 \cdot 10^{-4}$, lipids $5.7 \cdot 10^{-3}$ M), the vesicles of phospholipids tend to aggregate around a partly reassembled network of clathrin. When the tri-

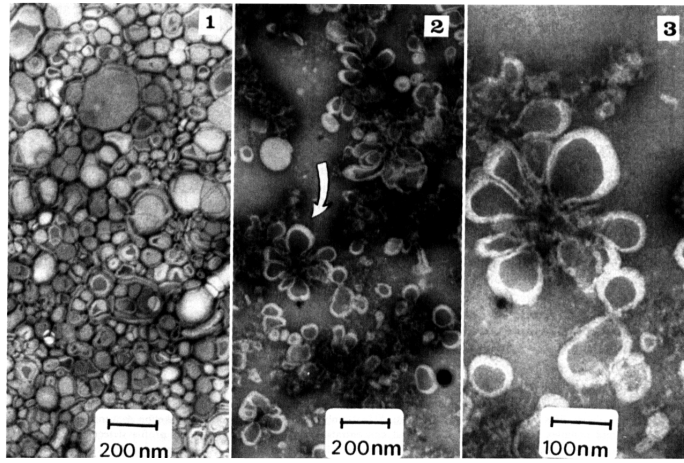

Fig. 2. Electron micrographs of negatively stained vesicles. (1) Control of egg PC vesicles (lipid concentration, $6.7 \cdot 10^{-3}$ M). (2) Reconstituted lipid-protein complexes (lipids, $5.7 \cdot 10^{-3}$ M; protein/lipid ratio $2.5 \cdot 10^{-4}$). (3) Enhanced magnification of the area shown in 2 by the arrow.

skelion/lipid ratio reaches $4 \cdot 10^{-4}$, these structures disappear. They are replaced by a simple adhesion of the proteins on the vesicle surface (chromatography results described above).

^{31}P-NMR

The interaction of clathrin coat proteins with purified lipids was also studied by ^{31}P-NMR (100 mM Mes or 10 mM Mes: triskelion/lipid ratio $4 \cdot 10^{-4}$ or $4 \cdot 10^{-3}$). The typical 121.4 MHz ^{31}P spectrum of DMPC small unilamellar vesicles above the transition temperature (30 °C) is well documented. It contains two resonance lines corresponding to the inside and the outside polar heads separated by 0.13 ppm (Fig. 3).

The binding of clathrin coat proteins induced two distinct effects.

There was first a line broadening which led to an unresolved resonance of 0.74 ppm width, slightly shifted towards low magnetic fields as compared to that of the outside polar heads. This line broadening confirms the size increase of the vesicles due to their fusion previously detected by chromatography (see above) and by light scattering (not shown). The rate of motion of the polar heads giving rise to this new resonance line was tested by T_1 relaxation measurements. Although it is known that with ^{31}P at 121.4 MHz chemical shift anisotropy and P–H dipolar interaction mechanisms contribute about equally to the behavior of T_1 [20], these measurements are generally interpreted in terms of rate of motion. Table I shows a complete study of T_1 as a function of the frequency assuming only one relaxation time. In the case of a single resonance line arising from only one component, the T_1 values should not vary with the frequency. As this is observed for DMPC small unilamellar vesicles, it can be deduced that both signals arising from inside and outside phospholipids have similar lattice relaxation times. Table I also shows the effect of protein addition on T_1 values. As no important perturbation occurred, it can be concluded that the rate of motion of the groups contributing to the spectrum reported in Fig. 3 is not greatly affected.

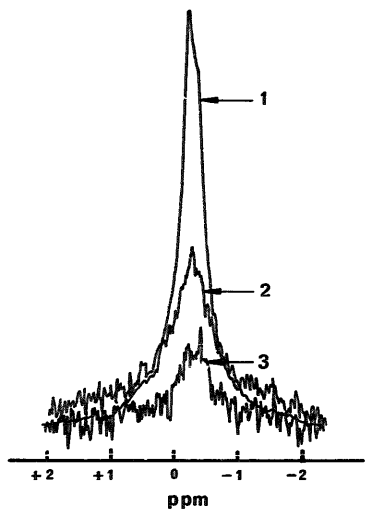

Fig. 3. Gated proton decoupled ^{31}P-NMR spectrum of DMPC small unilamellar vesicles. 1, Pure DMPC vesicles (lipids, $5 \cdot 10^{-3}$ M; 100 mM Mes, pH 6.5; 14000 scans). 2, Reconstituted lipid-protein complex (lipids, $5 \cdot 10^{-3}$ M; protein/lipid ratio, $4 \cdot 10^{-4}$; 100 mM Mes, pH 6.5; 15000 scans; spectrum magnification, 2). 3, Reconstituted lipid-protein complexes (lipids, $0.9 \cdot 10^{-3}$ M; protein/lipid ratio, $4 \cdot 10^{-3}$; 10 mM Mes, pH 6.5; 10000 scans; spectrum magnification, 4).

TABLE I

Behavior of the spin-lattice T_1 relaxation time of ^{31}P of DMPC as a function of the frequency

Frequencies are relative to the signal of the outer polar head.

Hz	Without clathrin		With clathrin	
	T_1 (s)	ΔT_1 (s)	T_1 (1)	ΔT_1 (1)
−40	–	–	0.91	±0.09
−35	–	–	0.98	±0.06
−23	–	–	1.08	±0.7
−16	1.12	±0.06	1.11	±0.06
−10	1.10	±0.05	1.13	±0.03
−4	1.17	±0.04	1.14	±0.05
+2	1.17	±0.04	1.14	±0.08
+14	1.10	±0.03	1.15	±0.06
+27	1.08	±0.06	1.13	±0.06

Secondly, there was an important loss in signal intensity. This could be evidenced by examining the first integral of the spectra. Under comparable experimental conditions (lipid concentration, number of scans, etc.) the intensity of the spectra recorded in the presence of proteins was only 30% of that of the spectrum recorded in their absence.

ESR experiments

Conventional ESR spectra of spin-labels included in small unilamellar vesicles and liposomes were recorded as a function of temperature. In order to minimize the spontaneous fusion of vesicles [21], the transitions were analyzed by decreasing the temperature. The hyperfine splitting, $A_{||}$, of DPLNO$^{\cdot}$ is supposed to reflect the flexibility of the hydrocarbon chains of the host membrane. As the temperature decreased, the spectrum showed a large change corresponding to the fluid-to-

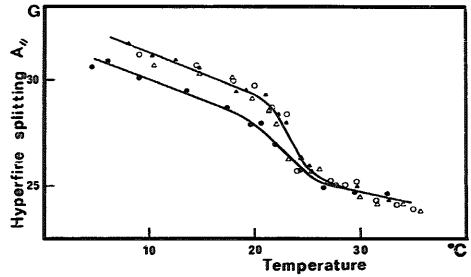

Fig. 4. Temperature dependence of the hyperfine splitting value for DPLNO˙ in different phospholipid-protein recombinants. ●, control of small unilamellar vesicles of DMPC; ▲, vesicles of DMPC and clathrin coat proteins (protein/lipid ratio, $4 \cdot 10^{-4}$); ○, control of liposomes of DMPC; △, liposomes of DMPC and clathrin coat proteins (protein/lipid ratio, $4 \cdot 10^{-4}$). Spin-label concentration in the membrane, 2%.

solid bilayer phase transition. The plot of $A_{//}$ versus temperature reported in Fig. 4 compares well with known data [22]. Addition of clathrin coat proteins to DMPC vesicles in 100 mM Mes (pH 6.5) did not change the hyperfine splitting of the fluid phase. But the transition, shifted towards higher temperatures, was more cooperative and the mobility in the solid phase was changed. All these modifications generated a new temperature prfile similar to that obtained for the DMPC liposomes, loaded or not with proteins.

ESR spectra of DPLNO˙ are thus mainly reporting the fusion of DMPC vesicles induced by clathrin coat proteins. The perturbation detected in ^{31}P-NMR experiments upon addition of the coat proteins is limited to the polar interface. The main phase transition of the chains of fatty acids in the inner part of the bilayers is not directly affected by the proteins.

As electrostatic interactions may contribute to the formation of the lipoprotein structure, we also incorporated partly anionic and cationic 5-doxyl derivatives and studied their hyperfine splitting as a function of temperature and ionic strength.

The incorporation of 5-doxylstearic acid in liposomes and vesicles of DMPC in 100 mM Mes (pH 6.5) is reported in Fig. 5. Due to its ionization, it has been shown that this compound gives two distinct ESR spectra at temperatures higher than 30°C [23]. As the apparent pK of the carboxylic group embedded in egg PC (6.2) is close to the pH of our solutions (6.5), each spectrum contributed almost equally. The temperature profile reported in Fig. 5 does not detail this situation. We verified that the binding of clathrin coat proteins does not change the ionization properties of the carboxylic acid. The $A_{//}$ values reported are the mean values

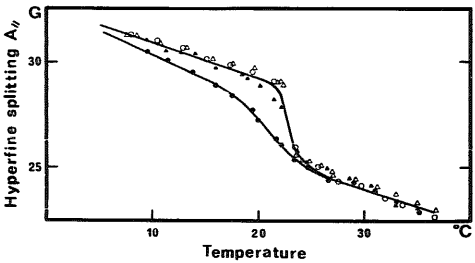

Fig. 5. Temperature dependence of the hyperfine splitting value for 5-AS in DMPC (100 mM Mes). ●, control of small unilamellar vesicles of DMPC; ▲, vesicles of DMPC and clathrin coat proteins (protein/lipid ratio, $4 \cdot 10^{-4}$); ○, control of liposomes of DMPC; △, liposomes of DMPC and clathrin coat proteins (protein/lipid ratio, $4 \cdot 10^{-4}$).

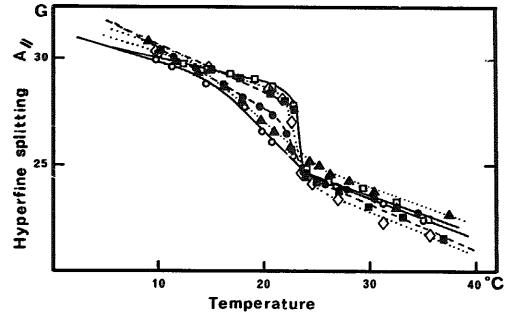

Fig. 6. Temperature dependence of the hyperfine splitting value for 5-AS in different phospholipid-protein recombinants (10 mM Mes, pH 6.5). ○, small unilamellar vesicles of DMPC; ●, reconstituted protein-lipid complexes (molar ratio, $4 \cdot 10^{-4}$); ▲, reconstituted protein-lipid complexes (molar ratio, $4 \cdot 10^{-3}$); □, liposomes of DMPC; ■, reconstituted protein-liposome complexes (molar ratio, $4 \cdot 10^{-4}$); ◇, reconstituted protein-liposome complexes (molar ratio $4 \cdot 10^{-3}$).

of the sum of the two contributions. As compared with the results reported on DPLNO˙, only slight differences in the hyperfine splitting values were observed (even in the absence of proteins). These differences have been explained by the fact that the depth of the 5-doxyl group in the membrane is dependent on the ionization state of the carboxyl group of the stearic acid [23]. Beside these differences and at that ionic strength, the effects due to fusion observed with DPLNO˙ were reproduced, but no particular effect due to the interaction of the polar part of the probe with the protein could be detected.

These interactions could only be evidenced after lowering of the ionic strength to 10 mM Mes pH 6.5 (Fig. 6). In this medium, the motion of the probe became less sensitive to the phase transition.

The same effect was observed, in 10 mM Mes (pH 6.5) with a cationic derivative (Fig. 7).

The interaction of the charged probes was further evidenced by the study of their lateral lipid diffusion.

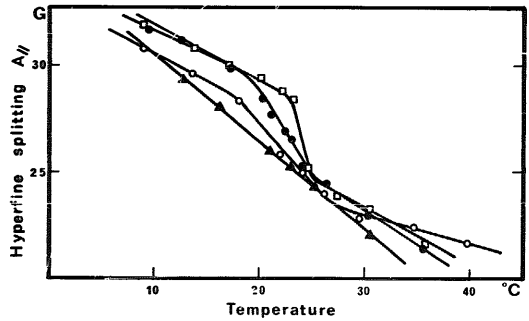

Fig. 7. Temperature dependence of the hyperfine splitting value for CAT 16 in different phospholipid-protein recombinants (10 mM Mes, pH 6.5). ○, small unilamellar vesicles of DMPC; ●, reconstituted protein-lipid complexes (molar ratio, $4 \cdot 10^{-4}$); ▲, reconstituted protein-lipid complexes (molar ratio, $4 \cdot 10^{-3}$); □, liposomes of DMPC.

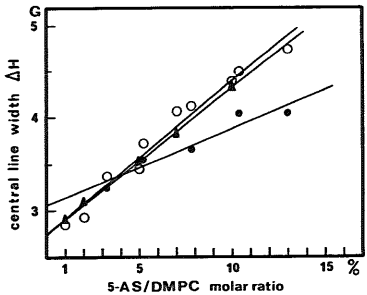

Fig. 8. Width of the central ESR line of 5-AS in DMPC liposomes at different 5-AS/DMPC molar ratios. ○, liposomes of DMPC; ▲, reconstituted protein-lipid complexes (molar ratio, $4 \cdot 10^{-4}$); ●, reconstituted protein-lipid complexes (molar ratio, $4 \cdot 10^{-3}$). Spectrometer settings were as follows: receiver gain, $2.5 \cdot 10^{5}$; modulation amplitude, 0.5 G. The central line was recorded in a scan range of 40 G to faciliate precise linewidth measurements. Temperature. 30 °C. Buffer 100 mM Mes (pH 6.5).

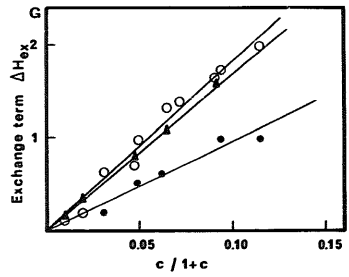

Fig. 9. Broadening of the central ESR line of 5-AS due to spin-exchange interactions. ○, liposomes of DMPC; ▲, reconstituted protein-lipid complexes (molar ratio, $4 \cdot 10^{-4}$); ●, reconstituted protein-lipid complexes (molar ratio $4 \cdot 10^{-3}$). The exchange broadening, ΔH_{ex}, is a measure of the probability of spin exchange. The diffusion constants have been deduced from the slopes of the straight lines and from parameters given by Sackmann et al. [21].

This was carried out in DMPC liposomes at 30 °C using different concentrations of 5-doxylstearic acid (Fig. 8). The short-range exchange interactions operating upon collisions between spin-label molecules cause a broadening of the central line of ESR spectra. The rate of lateral diffusion was deduced from the diffusion model and the numerical factors given by Sackmann et al. [24] (Fig. 9). The slope of the straight line obtained by plotting the exchange broadening ΔH_{ex} versus $c/(1+c)$ yielded $D_{diff} = 4.5 \cdot 10^{-8}$ cm^2/s for pure lipids (c, lipid

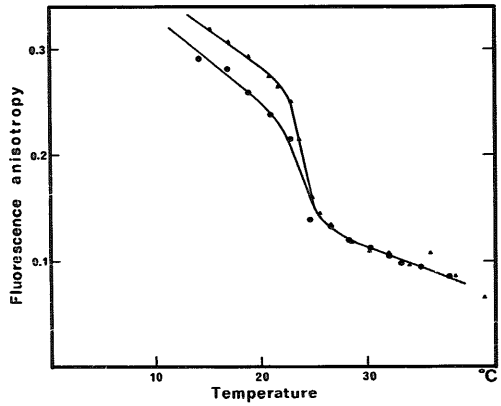

Fig. 10. Temperature dependence of the fluorescence anisotropy of DPH in DMPC. ●, control of small unilamellar vesicles of DMPC; ▲, vesicles of DMPC and clathrin coat proteins (protein/lipid ratio, $4 \cdot 10^{-4}$) (λ_{ex} = 362 nm; λ_{em} = 420 nm).

concentration). Parallel experiments yielded $D_{diff} = 4.3 \cdot 10^{-8} cm^2/s$ at a molar ratio of triskelion/lipid of $4 \cdot 10^{-4}$ and $D_{diff} = 3.0 \cdot 10^{-8}$ cm^2/s at a molar ratio of triskelion/lipid of $4 \cdot 10^{-3}$.

Our measurements imply the absence of any free probe. This was ascertained by careful analysis of all spectra. They also imply a uniform contribution of all spin labels. This requires that all labels are in equivalent positions (this is probably not exactly the case, because there is an inside/outside phospholipid distribution and because there are two ionic forms of the spin labels [23]). The values given above, thus, are only indicative, and more sophisticated methods can be employed [25]. Nevertheless, it is evident that the lateral mobility of the probe in the fluid phase of the membrane is significantly reduced only at a protein/lipid ratio close to that found in coated vesicles. It should be noted that Alfsen et al. [26] detected a lower diffusion coefficient of pyrene in coated compared with uncoated bovine brain vesicles.

Steady-state fluorescence anisotropy

The dynamics of the fatty acid chains was studied by inserting a non-anchored fluorescent probe, DPH. Its steady-state fluorescence anisotropy in small unilamellar vesicles of DMPC was measured between 15 and 40°C. Fig. 10 shows the phase transition from the gel to the liquid-crystalline state of the lipids in which 1% of probe molecules have been introduced. It was ascertained, with unlabelled DMPC, that, under our experimental conditions (see Materials and Methods), scattered light was negligible.

For pure DMPC vesicles, the transition was broad and centered at 23.7°C. This result agrees well with previously reported results [27].

When clathrin coat proteins were added to the phospholipids (protein/lipid ratio, $4 \cdot 10^{-4}$, 100 mM Mes), the fluorescence anisotropy of DPH was increased in the gel state but kept unchanged in the liquid-crystalline state, while the transition between the two states became more cooperative ($T_m = 24°C$).

As there is no preferential partition of DPH between phases of different microviscosity (in contrast with parinaric acid [8]), the presence of such phases would have been detected in our experiments. This is not the case, probably because the perturbations detected by ^{31}P-NMR upon addition of coat proteins are limited to the polar interface. In the absence of time-resolved measurements, however, it should be kept in mind that steady-state anisotropy data are not completely adequate for a detailed description of the rates of motion of DPH. Changes in the steady-state anisotropy of DPH may not only result from changes in the rotational diffusion of the probe, but also from changes in fluorescence lifetimes [29], or both.

Discussion and Conclusion

The work presented here focuses on the analysis of the perturbation of the phospholipid bilayers upon clathrin coat proteins binding under precise experimental conditions: synthetic lipids devoid of intrinsic proteins and the presence of clathrin accessory proteins. It was verified by electron microscopy that, under these conditions, the proteins do not restore around sonicated vesicles the characteristic polygonal network found around natural vesicles.

Binding to DMPC vesicles

It has been shown previously that clathrin coat proteins are able to form a stable complex with DOPC vesicles [19] and to induce a pH-dependent fusion of DOPC vesicles [19] and of phosphatidylserine containing vesicles [16]. But, at the present time, precise data concerning the number of sites and the binding constants of clathrin coat proteins for phospholipid bilayers are not available. This is the reason why, under our experimental conditions, the binding of clathrin coat proteins to uncharged lipids was tested by chromatography on Sephacryl S-1000 and completed by ^{31}P-NMR and ESR. In the chromatography experiment, egg PC phospholipids were substituted to DMPC, because the column elution was run at 20°C. At this temperature, the natural fusion of DMPC cannot be avoided. ^{31}P-NMR, ESR and detection of the chromatography elution by fluorescence revealed that, except for a low amount of protein, the clathrin binding to lipids is complete. The small amount of protein unable to form coats or to bind to lipids may have been denatured during the extraction process. Its presence does not introduce a large distortion in the expected stoichiometry of the complexes studied.

In addition to the protein/lipid complex formation, clathrin coat proteins induce the fusion of small unilamellar vesicles. While it is well documented that other proteins have the same effect, the corresponding mechanism is not completely clear. One essential condition for the induction of fusion, however, has been found in the bilayer destabilization after vesicle aggregation [30]. Portis et al. [31], for example, suggested that this could be achieved by the formation of cation-phospholipid complexes and Gad et al. [32] proposed an amplification of the phenomenon by polypeptides. Under our experimental conditions, Ca^{2+} is not present, DMPC vesicles are not charged and the destabilization is not apparent on the analysis of the thermal transitions (see below), probably because our observations are made on products after fusion. Nevertheless, some aspects of the fused vesicles by clathrin can be outlined. For example, the position of the peaks obtained after chromatography of the different particles indicates that the mean diameter of the fused particles is approx. 500 Å. This

leads to the conclusion that the fusion by clathrin induces a product slightly smaller than that obtained in the spontaneous fusion of pure DPPC when the lipids are incubated at 4°C for 7 days [33]. In this last case, a product of 750 Å is obtained which requires the combination of about 20 small unilamellar vesicles, while only ten would be combined in the presence of clathrin. In addition, it is apparent from the width at half height of the peak of chromatography that the fusion product is rather homogeneous. It does not contain traces of large liposomes, which could be excluded in the void volume of the column. The single temperature transition observed by ESR or steady-state fluorescence anisotropy measurements indicates that all the vesicles have fused. Consequently, in the absence of cooperative binding of the triskelions, our end-product must contain about 12 triskelions (at a protein/lipid of $4 \cdot 10^{-4}$) or 120 triskelion per vesicles (at a protein/lipid ratio of $4 \cdot 10^{-3}$). This is, respectively, about three times less and three times more than that found in native coated vesicles [34].

Gradient of lipid perturbation upon clathrin binding

^{31}P-NMR was used to study the modification of the polar interface of uncharged synthetic phospholipids after the binding of clathrin coat proteins. Two effects were seen which were due to two distinct populations of polar heads. First, the line broadening was originating from unaffected polar heads included in particles whose diameter was increased by the fusion process. Secondly, there was a loss of intensity, characteristic of polar heads whose rate of motion has been decreased.

These two effects can be rationalized as follows. The residual signal probably arises from the inner polar heads whose environment has not been changed (except the global rotational diffusion of the vesicle in which they are inserted). The loss of signal corresponds to the outer polar heads directly or indirectly affected by the proteins.

This interfacial interaction is not detected deeply in the membrane. The 5-doxyl group of DPLNO$^+$ (located below the glycerol backbone of the bulk lipids) only detects changes associated to the fusion process. When the clathrin coat proteins are added to small unilamellar vesicles, this probe shows that the temperature profile of the solid-to-liquid-crystalline transition of the chain of fatty acids is shifted to a higher value and that the process becomes more cooperative. The new profile corresponds to that of liposomes with or without proteins. In the solid state of DMPC and in the surrounding of the 5-doxyl group, the increase of order is, thus, only due to changes in orientation, packing and lateral pressure following the fusion process. Evidently, the vesicles fused by the clathrin coat proteins bear only some resemblance to liposomes and the assemblies which are formed cannot be strictly identical to liposomes. This only implies that the probe does not detect the difference between fused vesicles and liposomes. The results obtained by ESR are not inconsistent with the previously discussed ^{31}P-NMR results. Firstly, because a partial immobilization on the NMR time scale can be undetectable on the ESR time scale. Secondly, because a partial immobilization of the polar heads does not imply an immobilization of the hydrocarbon chains of the phospholipids.

A recent study indicates that when the nitroxide group substitutes the 5 position of a stearic acid, it is located in the membrane at a distance which is closer to the surface than the analogous acyl chain position of a phospholipid [35] and which can vary with the ionization state of the carboxylic acid [23]. By measurements of ^{13}C spin-lattice relaxation time enhancement, the dye has been found in the vicinity of the glycerol backbone. Taking into account the pH chosen in this work, the dye can migrate and probe the glycerol backbone environment of the inner and the outer phospholipids of our vesicles.

Even embedded in this new environment, the changes detected by the probe upon protein addition are not due to their binding, but to the fusion process. All the results, thus, attest that the motion and the thermotropic transition of the hydrocarbon chains of the uncharged phospholipids are not affected by the proteins. Any penetration of the proteins inside the hydrophobic core of the phospholipids can be excluded. More precisely, the clathrin coat proteins which are considered as extrinsic are not included in the membrane during the fusion process.

That the anionic derivatives can interact rather strongly with some charged groups of the protein has been demonstrated in two ways. First, the lateral diffusion of the stearic acid derivative is progressively lowered when more proteins are added (even in 100 mM Mes, pH 6.5). Secondly, there is a disordering effect of the proteins when the ionic strength is decreased and the dye does not detect the thermotropic transition of the hydrocarbon chains of the bulk phospholipids. The effect of low ionic strength can be even more pronounced on a cationic derivative whose nitroxide is also in the vicinity of the glycerol backbone [35], but whose migration towards the inner layer of the liposomes is excluded.

In conclusion, the binding of clathrin coat proteins to uncharged phospholipids devoid of intrinsic proteins lowers the rate of motion of the polar head-groups and induces the 'anchoring' of charged groups. These two effects are the result of interactions established at the membrane surface. In the inner core of the membrane, the flexibility of the hydrocarbon chains (hyperfine splitting of ESR spectra of doxyl groups) and the rotational diffusion of free dyes (fluorescence anisotropy of DPH) are not perturbed.

Model membranes and perspectives for native clathrin-coated vesicles

The extraction procedure used in this work delivers not only the clathrin triskelions but also the accessory coat proteins [12]. Much additional work is needed to attribute the 'anchoring' effect to one of the two groups of proteins. The work of Vigers et al. [8], however, has demonstrated that the 100 kDa proteins lie in close contact with the membrane of the coated vesicles. The extension of our work to the native system will determine whether this group is responsible for the 'anchoring' effect and whether this effect is intended for specific phospholipids or for the trapping of intrinsic proteins in the coated pits.

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