Interaction of clathrin with liposomes: pH-dependent fusion of phospholipid membranes induced by clathrin

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Clathrin, dissociated from coated vesicles of bovine brain and purified by gel chromatography, was found to interact with the lipid bilayer as shown by the spontaneous release of encapsulated fluorescent dye in liposome. Clathrin-induced dye release was enhanced at acidic pH in phosphatidylserine-containing vesicles. A strong correlation between dye release and fusion of liposomes was observed. In general, when there was a fast release of encapsulated dye induced by clathrin, a pH-dependent, clathrin-induced fusion was observed. Clathrin did not induce either dye release or fusion of egg phosphatidylcholine liposomes. The self-association of clathrin at low pH diminished the fusogenic activity. Fusion induced by clathrin at low pH could be stopped at pH above 5.0 and resumed by lowering the pH below 5.0. This suggests that the interaction of clathrin with phospholipid membranes can be regulated by pH.

Clathrin *Liposome* *Membrane fusion* *Phospholipid*

1. INTRODUCTION

Clathrin is the major coat protein forming the coat structure in both coated pits and coated vesicles in cells [1,2]. The purified assembly unit of the polygonal coat structure is now referred to as triskelion [3,4]. A triskelion is a molecular complex of 630 kDa that contains essentially 3 heavy (~180 kDa) and 3 light ((33–36 kDa) chains [3–6]. Triskelion dissociates from coated vesicles at pH above ~7.5 [7–9]. Clathrin can self-associate to form empty, cage-like polygonal coats when the pH is reduced to below 7 [7–10]. The coat protein is not considered an integral membrane protein, but, rather, a peripheral structure protein loosely associated with the vesicle membrane. The mechanisms of the assembly of coat structure in coated pits and the disassembly of the coat protein from the membrane of coated vesicles are not clearly understood. It has been suggested that an 'uncoating ATPase' uses ATP hydrolysis to release clathrin from coated vesicles [11]. A study of the interaction between clathrin and a major membrane component, phospholipid, is essential for a systematic approach to understanding the structure and the assembly mechanism of coated membranes. Recently, it has been shown that clathrin coat protein is able to form a stable complex with synthetic phosphatidylcholine (PC) and to induce leakage and pH-dependent fusion of dioleoylphosphatidylcholine (DOPC) vesicles [12,13]. Here, we have investigated the lipid specificity of the interaction of coat protein with phospholipid vesicles composed of various natural phospholipids. In this report we present evidence that coat protein indeed nonselectively induces the release of aqueous content of liposomes. We also found that the association of clathrin with liposomes containing phosphatidylserine (PS) at pH < 5.5 can promote fusion in these liposomes in the absence of divalent cations.

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2. MATERIALS AND METHODS

Coated vesicles from bovine brain were prepared according to [9]. Clathrin was dissociated from the coated vesicles by incubating the vesicles in 10 mM Tris-HCl, pH 8.8, and then purified on a Sepharose CL-4B column in 0.5 M Tris-HCl/0.05 M Mes/0.5 mM EDTA/0.025 mM $MgCl_2$/0.01% NaN_3, pH 7.5 [4]. The purified clathrin was then dialyzed at 4°C against 5 mM Tes/100 mM NaCl/0.1 mM EDTA, pH 7.4 (henceforth called liposome buffer). The protein concentration was determined according to Bradford [14] using bovine serum albumin as a standard. The purified preparation of clathrin was analyzed on SDS-polyacrylamide gel electrophoresis as described [15].

N-(7-Nitro-2,1,3-benzoxadiazol-4-yl)phosphatidylethanolamine (NBD-PE), *N*-(lissamine)rhodamine B sulfonyl)phosphatidylethanolamine (Rh-PE), PS, egg PC, DOPC and PE (transphosphatidylated from egg PC) were purchased from Avanti Polar Lipids (Birmingham, AL). Cholesterol from Sigma (St. Louis, MO) was recrystallized in methanol twice before use. Calcein was obtained from Hach (Ames, IA) and purified on a Sephadex LH-20 column. Calcein (2 g) was dissolved in 10 ml of 1 N NaOH and loaded on the column (2.5 × 50 cm) preequilibrated with eluting solution, 1 mM Na_2CO_3. Calcein was collected from the second band that followed the front nonfluorescent band. Calcein precipitate was obtained by adjusting the pH of the aqueous solution to 2. The concentration of calcein was determined spectrophotometrically using 7.0×10^4 as the molar extinction coefficient at 492 nm.

Large unilamellar vesicles (~1000 Å) were prepared by a reverse-phase evaporation technique, and the vesicles were extruded through a polycarbonate membrane (Nucleopore, Pleasanton, CA) with a pore size of 0.1 μm [16]. Calcein vesicles were prepared in 60 mM calcein at pH 7.4. The non-encapsulated calcein was removed from the liposome suspension by gel filtration on a Sephadex G-75 column (elution buffer, 5 mM Tes/100 mM NaCl/0.1 mM EDTA, pH 7.4). Lipid phosphorus analyses were made to determine the phospholipid concentration [17].

Aggregation of vesicles or protein was followed by the change in absorbance at 360 nm in a spectrophotometer. Release of vesicle content was measured as the increase of calcein fluorescence as the self-quenched calcein leaks out of the liposomes. The excitation wavelength was 430 nm, and the emission above 520 nm was measured by using a Corning 3-68 cut-off filter in an SLM-4000 fluorometer. Fusion was monitored by the resonance energy transfer technique [18,19]. Labeled liposomes containing both NBD-PE and Rh-PE at 1 mol% each were mixed with unlabeled liposomes at a mole ratio of 1:9 for the fusion measurement. The NBD fluorescence at 530 nm was recorded continuously while the sample was excited at 475 nm. Fusion was indicated by the increase of NBD fluorescence due to a decrease in the efficiency of resonance energy transfer between NBD and Rh resulting from the dilution of the fluorescent lipids into unlabeled liposomes. The total volume of each sample was 1 ml containing 50 nmol phospholipids, unless otherwise specified. Aggregation and fusion of liposomes were carried out at 25°C.

3. RESULTS AND DISCUSSION

The predominant polypeptide in the purified clathrin fraction shown on SDS-polyacrylamide gel electrophoresis was 180 kDa. Other polypeptides were seen in the 100–120, 52 and 30–36 kDa regions (fig.1). These polypeptides were used for the following experiments on clathrin-liposome interactions. Since the association of protein to lipid bilayers frequently alters the bilayer integrity and changes the permeability of lipid bilayers, the interaction of clathrin with phospholipid membranes can be easily examined by measuring the release of the encapsulated fluorescent dye, calcein, from the liposomes. There was an instantaneous release of calcein upon the addition of purified clathrin to the PS-containing liposomes. In contrast, there was no detectable change of permeability in egg PC liposomes. The rate of clathrin-induced release of calcein was proportional to the amount of clathrin present (fig.2). The leakage rate was clearly dependent on the lipid composition of liposomes at pH 7.4. In PS-containing liposomes, the leakage rate was affected not only by the amount of PS in the liposomes, but also by the accompanying lipid in liposomes. For instance, the leakage rate for

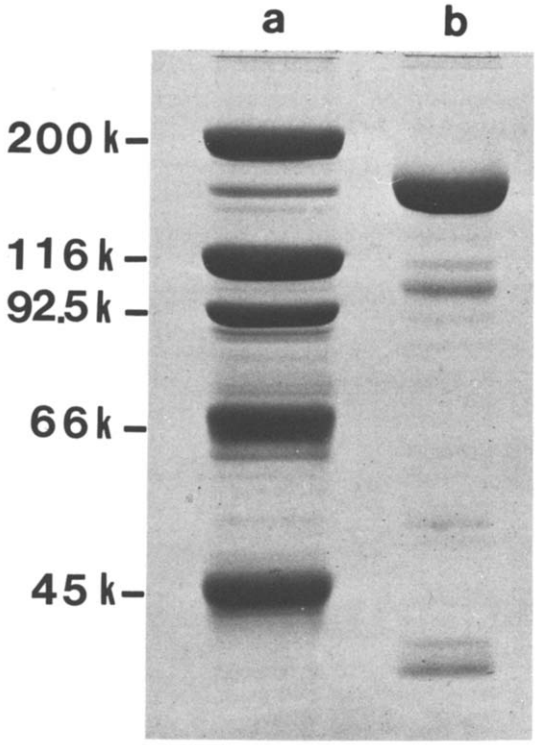

Fig.1. SDS-polyacrylamide gel electrophoresis of purified clathrin. SDS-polyacrylamide gels shown were 7.5% acrylamide and 10 cm in length. Gels were stained with Coomassie blue. Lanes: (a) molecular mass standards, (b) purified clathrin (10 μg).

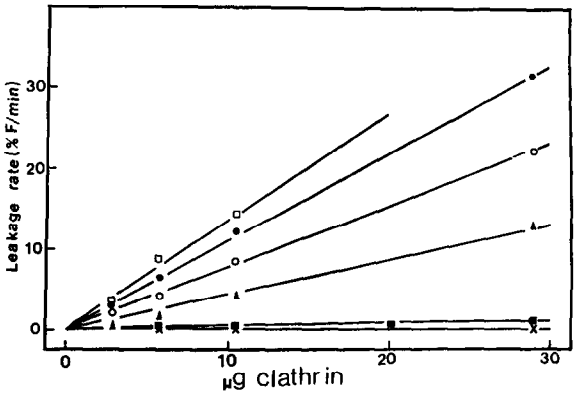

Fig.2. Clathrin-induced leakage of liposomes at pH 7.4. PC (×), PS/PE (1:2) (■), PS/PC (1:2) (▲), PS/cholesterol (2:1) (○), PS/PE (2:1) (●) and PS/PC (2:1) (□) liposomes encapsulating calcein were mixed with clathrin. The increase of fluorescence intensity due to dequenching of calcein outside the vesicles was measured at 25°C.

PS/PC (1:2) liposomes was much higher than for PS/PE (1:2) liposomes, as shown in fig.2. Based upon these leakage results, it seems that at neutral pH, PS is required for the clathrin-lipid interaction. In addition, clathrin appears to detect the structural difference between PS/PE (1:2) and PS/PC (1:2) liposomes, if the leakage rate reflects the extent of protein penetration into lipid bilayers. The small head group and the ability of PE to form intermolecular hydrogen bonding permit PE to pack more tightly than PC in lipid bilayers. Thus, it is more difficult for a protein molecule to penetrate PE-containing bilayers than PC-containing ones.

Since acidic pH has a strong effect on clathrin structure, it is important that the effect of pH on clathrin-induced leakage be investigated. A representative finding is illustrated in fig.3. The pH effect on calcein release of liposomes independent of clathrin was also measured for control: no more than 3% maximum fluorescence per min was obtained over the pH range 4–9. The marked enhancement in clathrin-induced release of calcein at lower pH was strongly dependent on lipid composition, and could be seen in all PS-containing liposomes, as shown in fig.3. There was no clear indication that clathrin interacted with egg PC liposomes at low pH. However, there was a fast release of calcein in DOPC liposomes upon the addition of clathrin at pH < 5.5. A similar result for liver clathrin has been described in DOPC

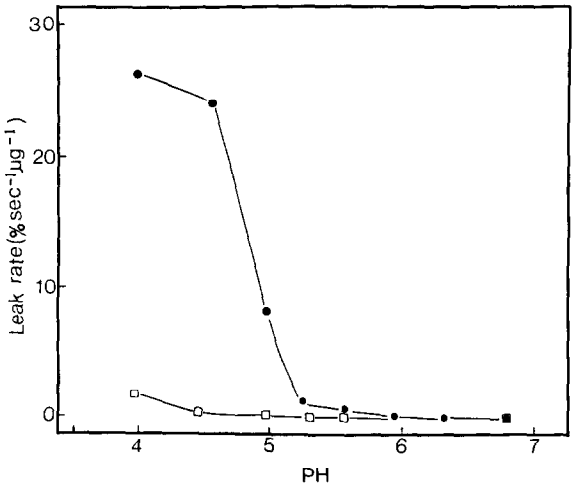

Fig.3. The effect of pH on clathrin-induced leakage of PS/PC (2:1) (●) and PC (□) liposomes.

liposomes [12]. It is noteworthy that clathrin at low pH is able to detect the structural difference between DOPC and egg PC. This suggests that the head group of PC is likely not the clathrin-binding site of PC liposomes. The clathrin-induced release of encapsulated dye in PS-containing liposomes is inhibited by divalent cations or polyamines [20], indicating that there is a charge-dependent interaction of clathrin with PS-containing liposomes.

To delineate the nature of the pH effect and the consequential change of liposome structure, we have examined clathrin self-association and aggregation of liposomes spectrophotometrically. In the presence of 50 μM egg PC, the turbidity change of clathrin in acidic medium was no different from that of clathrin alone, indicating that there was no detectable structural change of egg PC liposomes beyond clathrin self-association (fig.4B,C). In contrast, the turbidity in PS-containing liposomes increased substantially higher than the intrinsic change of clathrin self-association. There was no increase in turbidity when clathrin was initially mixed with liposomes at neutral pH. However, upon lowering the pH of the medium, a fast increase of turbidity was observed and most of the increase was not reversible by raising the pH back to 7.4 (fig.4D). In the case where liposomes were added to acid-induced self-associated clathrin, the rate of liposome aggregation appeared to be lower, and the extent of liposome aggregation could mostly be reversed by raising the pH to 7.4 (fig.4A). Nevertheless, both cases had substantial irreversible changes of turbidity, suggesting that, due to fusions, liposomes transformed into a larger structure. For controls, pH changes within the range 4.0–9.0 in liposomes alone did not have any measurable effect on liposome aggregation.

In view of the large clathrin-induced change of liposome structure described above and the fusion of DOPC liposomes mediated by clathrin at low pH [13], the lipid specificity of clathrin-induced fusion of liposomes was examined. Resonance energy transfer between NBD-PE, as an energy donor, and Rh-PE was used to quantify membrane fusion. Unlike DOPC, we found that there was no decrease in resonance energy transfer in egg PC liposomes in the presence of clathrin at various pH values. This suggests that clathrin does not induce fusion of egg PC liposomes even though DOPC liposomes can undergo pH-dependent clathrin-

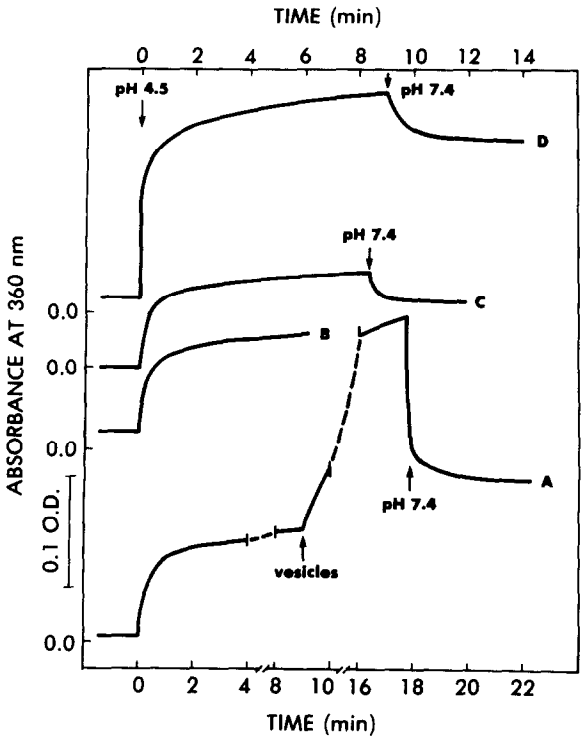

Fig.4. The effect of pH on clathrin self-association and clathrin-induced liposome aggregation. The pH of clathrin with or without liposomes was lowered to 4.5 by injecting 10 μl of 1 M acetic acid into 1 ml sample at time zero. Each sample contained 80 μg clathrin and 50 μM phospholipid. pH 7.4 was achieved by injecting 15 μl of 1 M Tris into the sample of pH 4.5. Clathrin was first induced to self-associate for 9 min at pH 4.5 followed by the addition of PS/PC (2:1) vesicles (A). A mixture of clathrin and PC liposomes (B), clathrin alone (C), and a mixture of clathrin and PS/PC/cholesterol (1:2:1) liposomes were subjected to pH changes.

induced fusion [13]. For PS/PC (1:2) liposomes, the fusion mediated by clathrin could be detected at pH 5.6, and the rate increased drastically when the pH was lowered further (fig.5). The lipid specificity of this pH-dependent, clathrin-induced fusion is illustrated in fig.6. Note that cholesterol seems to have no effect on clathrin-induced liposome fusion. Fusion of PS-containing liposomes induced by clathrin at pH < 5 can be arrested at any level when the pH is raised above 5, and can be resumed by lowering the pH again (fig.7A). In contrast to a spontaneous resumption of turbidity increase, there is a lag time for fusion

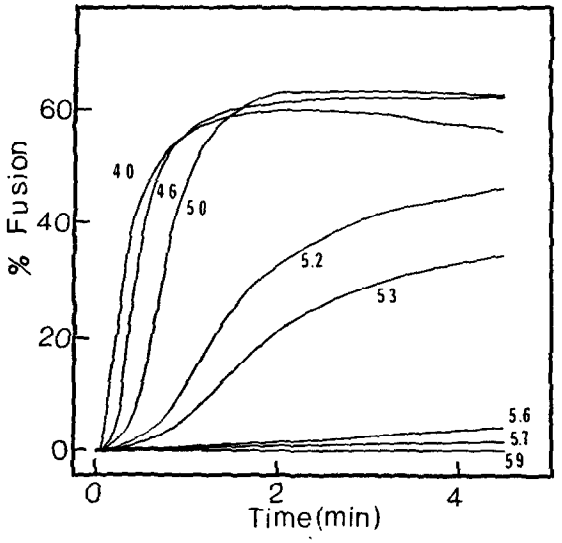

Fig.5. pH dependency of clathrin-induced fusion of PS/PC (1:2) liposomes.

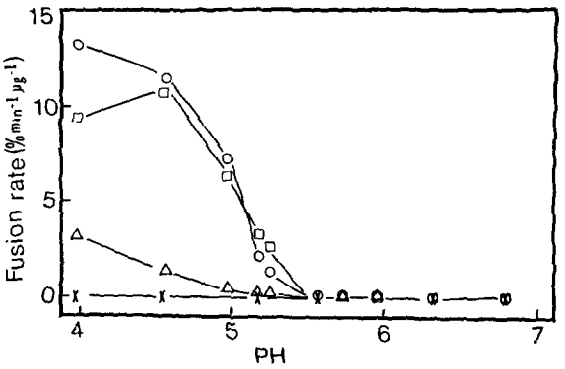

Fig.6. The effect of pH on the rate of clathrin-induced fusion of PC (×), PS/cholesterol (2:1) (△), PS/PC (1:2) (○), and mixture of PS/PC (1:2) and PS/PC (2:1) (□) liposomes.

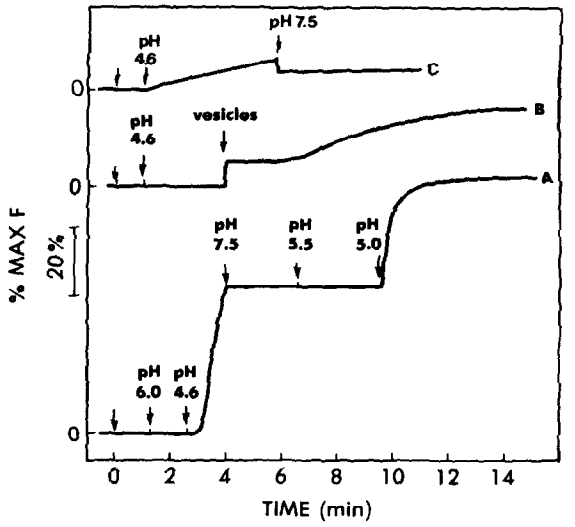

Fig.7. (A) pH-dependent clathrin-induced fusion of PS/PC liposomes. The pH was changed by adding a small volume of either 1 M acetic acid or 1 M Tris. Each sample contained 50 μM phospholipid composed of 1 part labeled PS/PC (1:2) liposomes and 9 parts unlabeled PS/PC (2:1) liposomes. Clathrin (20 μg) was mixed with liposomes at neutral pH at time zero. The mixture was then subjected to pH changes. (B) Clathrin (40 μg) was induced to self-associate for 4 min before liposomes were added. (C) pH-dependent clathrin-induced fusion of PS/PE (1:2) liposomes. Clathrin (40 μg) was added to liposomes and the mixture was subjected to pH changes.

to take place upon acidification. The fusogenic activity of self-associated clathrin was diminished irreversibly (fig.7B) even though its aggregation capacity for liposomes remained high (fig.4A). It should be noted that unlike the aggregation reaction there was an obvious lag time before fusion took place (fig.7).

A close examination of the leakage, aggregation and fusion of liposomes induced by clathrin reveals that these actions are strongly dependent on the pH. Since there is no detectable change of liposomes free of clathrin at low pH, any structural change of liposomes must come from the interaction of clathrin with the phospholipid membranes. At neutral pH, clathrin triskelion causes release of encapsulated calcein in PS-containing liposomes, but not aggregation of these liposomes. Individual triskelion only appears to associate with a liposome, and such a triskelion-associated liposome is not able to overcome the electrostatic repulsion between adjacent liposomes.

It is known that an increase in quantum yield or a blue shift of some fluorophores is an indication of fluorophore binding to a hydrophobic region of a protein [21]. By using this technique, a pH-dependent increase of hydrophobic exposure of clathrin was found to correlate with both the binding of clathrin to liposomes and clathrin's fusogenic capability (T. Yoshimura, in preparation). Therefore, upon acidification, 2 events can

occur in clathrin-liposome suspension. (i) Clathrin undergoes conformational change, and larger or multi-hydrophobic sites are then available for liposome-clathrin interactions. Thus, the leakage rate of liposomes increases due to larger hydrophobic penetration by liposome-associated triskelion; the aggregation of liposomes is facilitated by protein-protein interactions of liposome-associated triskelions. (ii) Lower pH facilitates the aggregation of liposomes due to the reduction of surface charge density. Furthermore, clathrin triskelion is large enough to bridge 2 adjacent liposomes through its multi-reaction sites even though there is still residual repulsion among liposomes. It appears that fusogenic activity of clathrin induced by low pH is not as fast as liposome aggregation, especially in the case of prepolymerized clathrin as shown in fig.7. Slow fusion occurs after more than 1 min lag time, indicating that most polymerized clathrin can only promote aggregation which then does not lead to fusion (figs 4A and 7B).

Even though we cannot rule out the possibility that the decrease of energy transfer is due to the exchange of the fluorescent probes promoted by clathrin in the aggregated systems, there are some observations which argue against this possibility. If the decrease of energy transfer arises from the exchange of probes during aggregation, the rate of the fluorescence increase should be parallel to the rate of aggregation regardless of the type of liposome used. Two experimental observations show the contrary. (i) There was no fluorescence increase during the lag time where liposomes had been already highly aggregated (cf. figs 4 and 7). In addition, there was an abrupt stop of the fluorescence increase while disaggregation was still proceeding. (ii) Under the same conditions, aggregated PS/PE (1:2) liposomes did not fuse as readily as PS/PC (1:2) liposomes (fig.7C).

Further examination of both the leakage rate and fusion induced by clathrin reveals that there is a strong correlation between the fusion susceptibility and leakage rate of liposomes composed of various phospholipids. For example, PS/PC (1:2) liposomes, which have a higher leakage rate than PS/PE (1:2) liposomes, fuse more readily under the same conditions. As for egg PC liposomes, neither fusion nor leakage induced by clathrin was observed. Since DOPC vesicles can be induced to fuse by clathrin at low pH, as shown by Blumenthal et al. [13] and in this study, it appears that the surface charge of liposomes is not a major factor for determining fusion susceptibility. A stronger hydrophobic perturbation by clathrin penetration into bilayers seems to be a major factor for facilitating the destabilization of closely apposed bilayers and the mixing of lipid components. The lower hydration of PE compared to PC [22] and its tendency to form nonbilayer structures [23,24] have been proposed to be determinants of the difference in Ca^{2+}-induced fusion of liposomes composed of acidic phospholipids and PE or PC [25]. The pH-dependent fusogenic activity of clathrin is not reduced by PC in PS/PC liposomes, as it is with Ca^{2+}. The molecular basis for this evident difference in fusion capacity between PS/PE and PS/PC is not readily identified. However, the fusion rate and the leakage change induced by clathrin under acidic conditions are well correlated. In general, when there is a fast release of encapsulated calcein induced by clathrin, a pH-dependent, clathrin-induced fusion is observed.

Clathrin triskelion consists of 2 major polypeptides, heavy chains and light chains. When the purified triskelion is exposed to pH below 4.5, light chains are released from the heavy chains [26]. In our preliminary experiment, the light chain released from the clathrin triskelion in this manner is found to bear no fusogenic activity. The purified heavy chain free of the light chain will be needed for disentangling the involvement of certain protein domains that interact with phospholipid bilayers and initiate the pH-dependent fusion.

The effect of pH on the interaction of clathrin with liposomes represents an interesting topic for studying protein-lipid interactions. However, the cytoplasmic pH is generally higher than the optimal pH for the clathrin-induced fusion of liposomes described here. Unfortunately, just as in all the in vitro studies on the pH-dependent assembly of clathrin baskets, the physiological significance of the pH-dependent fusogenic activity of clathrin in vivo remains to be determined.

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