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Sequence of critical events involved in fusion of phospholipid vesicles induced by clathrin

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Membrane fusion induced by clathrin is accompanied by several events such as conformational change, membrane binding and association of clathrin, and membrane aggregation (Maezawa et al. (1989) Biochemistry 28, 1422–1428; Maezawa and Yoshimura (1990) Biochem. Biophys. Res. Commun. 173, 134–140). To clarify the sequence of these events, we examined their time-courses by reducing the pH of the medium from 7.4 to a given pH in the range of 3.5–5.0 at 25°C or 10°C. Large unilamellar vesicles composed of phosphatidylserine and phosphatidylcholine were used in most experiments. The half-time for conformational change of clathrin was less than those for membrane binding and association of clathrin. The half-times and the initial rates of membrane binding and association of clathrin were similar order of magnitude, although the pH-profiles of the initial rates of the two events were somewhat different. Membrane aggregation started after membrane binding of clathrin. A lag phase was observed in the time-course of membrane fusion, whereas there was no lag phase in membrane binding and association of clathrin and membrane aggregation. Moreover, the lag time before fusion was independent of the clathrin concentration, although the initial rates of these three events were dependent on it, suggesting that the three reactions are not responsible for the lag phase before fusion, and that there is some other event(s) in the lag time. On the other hand, there was a threshould-pH in the pH profile of the lag-time and the threshold-pH coincided with the critical pH at which the final associated state of clathrin was apparently reversed in the presence and absence of liposomes, suggesting that the event(s) in the lag phase may be related to this final associated state of clathrin molecules on the liposome membranes. These results indicate that clathrin-induced fusion of liposomes is initiated through the following sequential events: conformational change of clathrin, membrane binding and association of clathrin, which occur simultaneously but independently, membrane aggregation, an event(s) in the lag phase, and actual fusion.

Introduction

Membrane fusion is a key event in many cellular activities, such as endocytosis, fertilization, membrane transport and virus infection. Recently, proteins have been recognized to play important roles in membrane fusion in vivo [1–3], and a lot of studies on the molecular mechanism of protein-dependent membrane fusion have been carried out in different aspects [4–9]. Results have suggested that membrane fusion is triggered through several events, and that fusion-active proteins are involved in various stages of the fusion process. Very recently, some models for the fusion intermediate have been proposed in the virus protein-mediated fusion process [10–12]. However, the sequences of the several events before membrane fusion is still speculative, because there is no suitable assay method for continuous monitoring of the events.

Clathrin is a major coat protein of coated pits and vesicles formed in receptor-mediated endocytosis [13]. Blumenthal et al. [14] and we [15] found that this

Abbreviations: ANM, *N*-(1-anilinonaphthyl-4)maleimide; BIPM, *N*-[*p*-(2-benzimidazolyl)phenyl]maleimide; DACM, *N*-(7-dimethylamino-4-methyl-3-coumarinyl)maleimide; LUV, large unilamellar vesicles; NBD-PE, *N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)phosphatidylethanolamine; PC, phosphatidylcholine; PS, phosphatidylserine; RET, resonance energy transfer; Rh-PE, *N*-(lissamine rhodamine B sulfonyl)phosphatidylethanolamine; standard buffer, 10 mM Tes buffer (pH 7.4) containing 100 mM NaCl, 0.1 mM EDTA, and 0.02% NaN_3; Tes, 2-{[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]amino}ethane sulfonic acid.

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soluble protein has membrane fusion activity at low pH. This prompted us to elucidate the general mechanism of protein-induced membrane fusion at a molecular level by characterizing the fusion reaction mediated by clathrin. Previously, we found that the fusion reaction is associated with conformational change, membrane binding and association of clathrin, as well as membrane aggregation [16–18]. In these studies, we detected these events using the following spectrophotometric indicators: the fluorescence intensity of ANM tagged to the clathrin molecule as an indicator of conformational change, RET from tryptophan in the clathrin molecule to NBD groups on the liposome membranes as a measure of membrane binding of clathrin, RET from BIPM to DACM separately tagged to the clathrin molecules as an indicator of association of clathrin, turbidity of the mixture of clathrin and liposomes as an estimate of membrane aggregation, and RET from NBD to Rh groups on liposome membranes as an indicator of membrane fusion. Using these methods, we could monitor all these events continuously. In the present study, therefore, we measured the time-courses of these events and determined their sequence. We found that membrane fusion was initiated after a lag phase, and so we also examined the relation of this lag phase to other events.

Materials and Methods

Materials

Bovine brain PS, egg PC, NBD-PE and Rh-PE were obtained from Avanti Polar Lipids Co. (Birmingham, AL). ANM, BIPM and DACM were purchased from Teika Seiyaku Co. (Toyama, Japan). All other reagents used were commercial products of the highest grade available.

Preparation of clathrin

Clathrin was purified from crude coated vesicles of bovine brain as described previously [16]. Purified clathrin in 0.5 M Tris-HCl buffer (pH 7.5) was stored at 0°C and dialyzed against 10 mM Tes buffer (pH 7.4) containing 100 mM NaCl, 0.1 mM EDTA, and 0.02% NaN_3 (standard buffer) before use. The concentration of clathrin was determined taking its absorption coefficient at 280 nm as $E^{1\%}_{1cm} = 10.9$ [19].

Fluorescence labeling of clathrin

Three types of fluorescent probe-labeled clathrins, ANM-, BIPM-, and DACM-labeled clathrin, were prepared by treating samples of 1–2 mg of clathrin with 5–8-fold molar excess of the three probes with respect to the clathrin heavy chain in 1 ml of standard buffer. After incubation at 0°C for 20–60 min, the reactions were stopped by addition of 6-fold excess of 2-mercaptoethanol, and the resulting solutions were dialyzed overnight against the same buffer at 4°C. The amounts of ANM, BIPM and DACM incorporated into the protein were determined spectrophotometrically to be 2.74, 2.37 and 2.58 mol per mol of clathrin heavy chain, respectively, using millimolar absorption coefficients of 10.8 mM^{-1} cm^{-1} at 345 nm for ANM-clathrin [20], 28.0 mM^{-1} cm^{-1} at 314 nm for BIPM-clathrin [21] and 19.8 mM^{-1} cm^{-1} at 380 nm for DACM-clathrin [22]. Labeling of clathrin with fluorescent probes had no effects on the fusion activity of clathrin.

Preparation of liposomes

LUV were prepared with PS and PS/PC(2:1) in standard buffer by reverse-phase evaporation [23] with the modifications described by Wilschut et al. [24] and then extruded through polycarbonate membranes of 0.1 μm pore size [25]. PS/PC(2:1) LUV labeled with both NBD-PE and Rh-PE at 1 or 0.1 mol% each and labeled with NBD-PE at 2 mol% were prepared by the same method. The vesicle concentration was determined by measuring total phospholipid phosphorus by the method of Bartlett [26].

Measurements of time-course of events

Continuous monitoring of all the events except conformational change of clathrin was carried out with a fluorescence spectrophotometer (Hitachi 650-60) or a double-beam spectrophotometer (Hitachi 624), equipped with a constant-temperature cell holder and stirrer. The reaction was initiated by rapid injection of a trace amount of 5 M acetic acid into 1 ml of solution with a microsyringe to adjust the pH to the required value.

A stopped-flow spectrophotometer (Otsuka Electric Co., RA-401), equipped with a fluorescence attachment (RA-414) and a constant-temperature cell holder, was used to monitor the time-courses of conformational change and membrane binding of clathrin. Samples in standard buffer were added to one compartment of the cell and acetate buffer to the other compartment to give a 1:1 mixture of the two of the desired concentration of clathrin and the desired pH.

Experiments were performed at final concentrations of clathrin and LUV of 15 μg/ml and 50 μM, respectively.

Assessment of events

Membrane fusion was assessed by RET assay using a mixture of LUV labeled with both NBD-PE and Rh-PE at 1 mol% each and unlabeled LUV in a molar ratio of 1:9 in the presence of clathrin [17]. The excitation and emission wavelengths used were 475 and 530 nm, respectively. The 100% fusion level was set at the fluorescence intensity of LUV containing the two fluorescence probes at 0.1 mol% each, which were prepared separately.

Conformational change of clathrin was assessed by measuring the fluorescence intensity of ANM groups bound to clathrin [17]. The excitation wavelength was set at 280 nm, and the fluorescence at a wavelength of over 300 nm was measured.

Membrane binding of clathrin was assessed by RET from tryptophan in the clathrin molecule to NBD groups in LUV, and the extent of the transfer was monitored with excitation and emission wavelengths of 275 and 339 nm, respectively, in a fluorescence spectrophotometer and with excitation and emission wavelengths of 280 and 300 nm, respectively, in a stopped-flow spectrophotometer, in the pH range of 3.8–5.0, the pH range in which the transfer reached a similar final level [17]. A correction was made for the contributions of membrane aggregation and fusion to fluorescence quenching by subtracting the value for change in intensity in a mixture of clathrin and unlabeled liposomes.

The associations of clathrin in the presence and absence of LUV were evaluated by RET from BIPM groups to DACM groups in an equimolar mixture of BIPM-clathrin and DACM-clathrin. Measurements were made with excitation and emission wavelengths of 320 and 470 nm, respectively. This procedure allowed us to assess close-contact of clathrin molecules, which was referred to as 'association' (see Results) [18].

Self-aggregation of clathrin molecules in the absence of liposomes was assessed by measuring light scattering of clathrin solution in a fluorescence spectrophotometer. For this measurement, the excitation and emission wavelengths were both set at 500 nm.

Liposome aggregation was assessed by measuring the turbidity of a mixture of clathrin and LUV at 360 nm.

Results

Time-courses of events involved in the fusion process

We have reported that membrane fusion induced by clathrin is a multi-step reaction involving conformational change, membrane binding and association of clathrin, membrane aggregation, and actual fusion [16–18]. To determine the sequence of the events involved in the fusion reaction, we measured the time-courses of the events in the pH region of 3.5–5.0, in which all added clathrin molecules bind to liposome membranes [17], at 25 and 10°C using PS/PC(2:1) LUV.

First, we measured the time-courses of fusion of liposomes by RET assay (Fig. 1). A lag phase was observed before the fusion reaction. The lag time depended on the pH and temperature: it increased with increase in pH and decrease in temperature. For example, at pH 4.01 the lag times at 25 and 10°C were 2 and 20 s, respectively, and at pH 4.72 the respective lag times were 13 and 65 s. The pH dependencies of the initial rate and the extent of fusion were similar to those described in our previous paper [17].

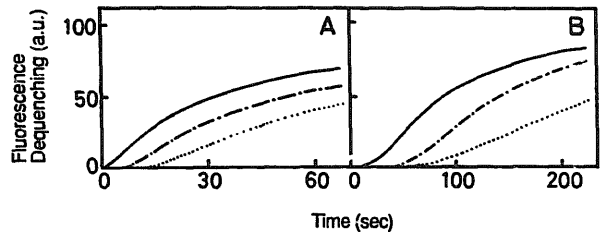


Fig. 1. Time-courses of fusion of PS/PC(2:1) liposomes at 25°C (A) and 10°C (B). A mixture of clathrin (15 μg/ml) and liposomes labeled with both NBD-PE and Rh-PE and unlabeled (total 50 μM) in 1 ml of standard buffer was put into a cuvette, a trace of 5M acetic acid was added to adjust the pH of the medium to pH 4.01 (———), pH 4.47 (·–·–·), or pH 4.72 (- - - - -), and dequenching of NBD fluorescence was monitored continuously. The excitation and emission wavelength were 475 and 530 nm, respectively.

Next, we measured the time-courses of membrane binding of clathrin at 25 and 10°C with fluorescence and stopped-flow spectrophotometers. The time-courses measured by these two spectrophotometers showed essentially the same pattern. Results with the fluorescence spectrophotometer are shown in Fig. 2A and B. The binding also depended on both the pH and temperature, but started immediately after the medium was reduced to an acidic pH: there was no lag phase. More than 80% of the clathrin bound to the membranes during the lag time before fusion.

The time-courses of membrane aggregation at 25 and 10°C are shown in Fig. 2C and D, respectively. The reaction profiles also depended on both the pH and

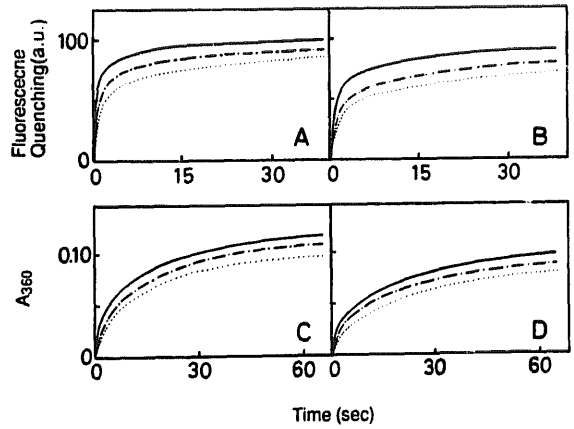


Fig. 2. Time-courses of membrane binding of clathrin (A, B) and liposome aggregation (C, D). A mixture of clathrin (15 μg/ml) and liposomes (50 μM) in 1 ml of standard buffer was put into a cuvette, and a trace of 5 M acetic acid was added to adjust the pH of the medium to pH 4.01 (———), pH 4.47 (·–·–·), or pH 4.72 (- - - - -). For A and B, quenching of Trp fluorescence due to increase in the efficiency of RET from Trp residues in clathrin to NBD groups on PS/PC(2:1) liposomes was monitored continuously at 25°C (A) or 10°C (B) with excitation and emission wavelengths of 275 and 339 nm, respectively. For C and D, the turbidity change of PS/PC(2:1) liposomes at 360 nm was measured continuously at 25°C (C) or 10°C (D).

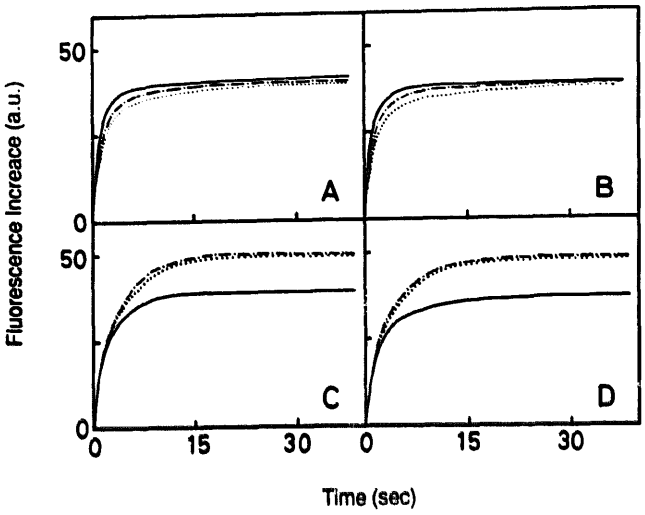


Fig. 3. Time-courses of clathrin association in the presence (A, B) and absence (C, D) of PS/PC(2:1) liposomes. A mixture of BIPM-clathrin and DACM-clathrin in molar ratio of 1:1 (total, 15 μg/ml) in the presence or absence of liposomes (50 μM) in 1 ml of standard buffer was put into a cuvette. A trace of 5 M acetic acid was added to adjust the pH of the medium to pH 4.01 (———) pH 4.47 (·–·–·), or pH 4.72 (- - - - -), and the fluorescence increase at 470 nm was measured with an excitation wavelength of 320 nm at 25°C (A, C) or 10°C (B, D).

temperature and showed no lag phase. The extent of membrane aggregation during the fusion lag could not be estimated, because the change in turbidity did not level off during the measurement, probably owing to the additional contribution of membrane fusion to the turbidity. However, liposome aggregation seemed to proceed to a considerable extent during the lag time before fusion.

Fig. 3 shows the time-courses of association of clathrin in the presence and absence of liposomes at 25 and 10°C. They showed no lag phase, and the reaction profiles depended somewhat on pH but scarcely on temperature. The fluorescence increases almost leveled off during the lag time before the fusion reaction. The pH-dependency of the final fluorescence level in the presence of liposomes was different from that in their absence.

We also measured the time-course of conformational change of clathrin using a stopped-flow spectrophotometer (data not shown). The reaction patterns varied with changes in pH and temperature, and there was no lag phase.

Comparison of kinetic parameters for conformational change, membrane binding, association and self-aggregation of clathrin

The above results clearly showed that there is a lag phase in the time-course of membrane fusion, whereas no lag phase in other events. However, since the time-courses of other events were similar, we could not determine the sequence of the events before fusion from the data. Therefore, we estimated the half-times ($t_{1/2}$) for conformational change of clathrin, binding of clathrin to liposome membranes, and association of clathrin at 25 and 10°C at four pH values (Table I). These values were calculated on the basis of data, some of which are shown in Figs. 2 and 3. The $t_{1/2}$ values for conformational change increased with increase in pH, and were in the ranges of 0.25–0.52 and 0.31–0.76 s at 25 and 10°C, respectively. The half-times for membrane binding and association of clathrin also depended somewhat on pH and slightly on temperature, but were always greater than 0.8 s. These results indicate that conformational change of clathrin occurs before both membrane binding and association of clathrin.

We next determined the initial rates of membrane binding of clathrin and association of the protein in the presence and absence of liposomes from the reaction patterns, some of which are shown in Figs. 2 and 3. Fig. 4 shows the pH-dependencies of the initial rates of these events. The pH-dependencies of the rates of membrane binding were different from those of clathrin association in the presence and absence of liposomes at both 25 and 10°C: the initial rate of membrane binding of clathrin decreased steeply with increase in pH, whereas that of association of clathrin in the presence and absence of liposomes changed only slightly with change in pH. These results indicate that the two events occur independently. However, the values of the half-times and the initial rates of these two events were similar order of magnitude (Table I and Fig. 4), indicating that membrane binding and association of clathrin are simultaneous events.

TABLE I

Half-times of conformational change of clathrin, binding of clathrin to PS/PC(2:1) LUV and association of clathrin in the absence of liposomes

Half-times for conformational change of clathrin were estimated from plots of change in ANM fluorescence against time. Half-times for membrane binding and association of clathrin were estimated from reaction patterns, some of which are shown in Figs. 2 and 3, respectively.

Temperature	pH	$t_{1/2}$ (s)		
		conformational change	membrane binding	association
25°C	4.01	0.25	0.8	0.9
	4.28	0.31	1.1	1.1
	4.60	0.49	1.6	1.1
	4.90	0.52	2.4	1.3
10°C	4.01	0.31	1.0	1.1
	4.28	0.44	2.3	1.3
	4.60	0.63	3.2	1.3
	4.90	0.76	5.4	1.4

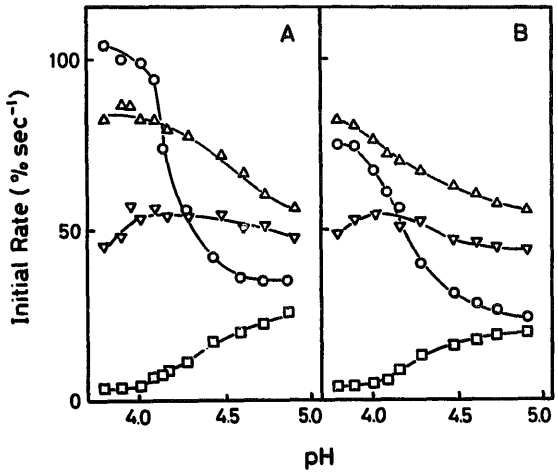


Fig. 4. pH dependencies of the initial rates of membrane binding, association and self-aggregation of clathrin at 25°C (A) and 10°C (B). The initial rates of membrane binding (○) and association of clathrin in the presence (△) and absence (▽) of liposomes were obtained from plots some of which are shown in Figs. 2 and 3, respectively. The initial rates of self-aggregation of clathrin (□) were estimated from the reaction pattern measured by light scattering at 500 nm.

We have previously demonstrated that clathrin molecules form insoluble self-aggregates in the absence of liposomes in the fusion-inducible pH region [16,17], which could be monitored by light scattering or turbidity measurement. We have also demonstrated assembly of clathrin molecules on liposome membranes by measuring RET for the mixture of BIPM-labeled and DACM-labeled clathrin [18]. This RET measurement allowed us to assess close-contact of the protein, which was referred to as 'association'. To clarify the relation between 'self-aggregation' and 'association' of clathrin molecules, we compared the initial rates for self-aggregation of clathrin with those for association of the protein in the absence of liposomes under the same conditions. As also shown in Fig. 4, the initial rates of clathrin self-aggregation increased with increase in pH, but were lower than those of clathrin association in the pH region examined, indicating that clathrin association, that is, close-contact of clathrin molecules, precedes formation of self-aggregates of the protein: in other words, in the absence of liposomes, small soluble assemblies composed of several clathrin molecules and then large insoluble aggregates are formed.

Relation of the fusion lag phase to other events

As shown in Fig. 1, clathrin-induced fusion of PS/PC(2:1) liposomes occurs after a time lag. We have previously shown that several events are involved in the membrane fusion process [16–18], suggesting that the lag phase must be controlled by some event before membrane fusion. As shown in Figs. 1–4, with increase in pH, the fusion lag-time increased and concomitantly the initial rates of membrane binding and association of clathrin, and liposome aggregation decreased. These findings imply that the fusion lag may be due to a delay of either of these three events. However, no lag phase was observed in the three events. We also examined the effect of the concentration of clathrin on the initial rates of membrane binding and association of clathrin and liposome aggregation, and on the fusion lag-time at pH 4.47 and 25°C. As shown in Table II, with increase in the concentration of clathrin, the initial rates of these three events increased, but the fusion lag-time did not change. These results suggest that these reactions are not responsible for the fusion lag-phase, and that there is some other event(s) in the lag phase. However, the pH-dependency of final level of the association of clathrin in the presence of liposomes was apparently different from that in the absence of them (Fig. 3). Thus, we thought that the final associated state of

TABLE II

Dependences of initial rates of membrane binding and association of clathrin and liposome aggregation and of fusion lag time on clathrin concentration

All measurements were carried out at pH 4.72 and 25°C in the presence of PS/PC(2:1) liposomes.

Clathrin (mg/ml)	Binding rate (% s^{-1})	Association rate (% s^{-1})	Liposome aggregation rate (A_{360} s^{-1})	Lag time (s)
4.7	12.6	42.8	0.008	11.9
10.3	28.7	51.7	0.013	12.0
15.0	35.0	61.0	0.017	12.8
18.2	48.3	73.4	0.024	11.7

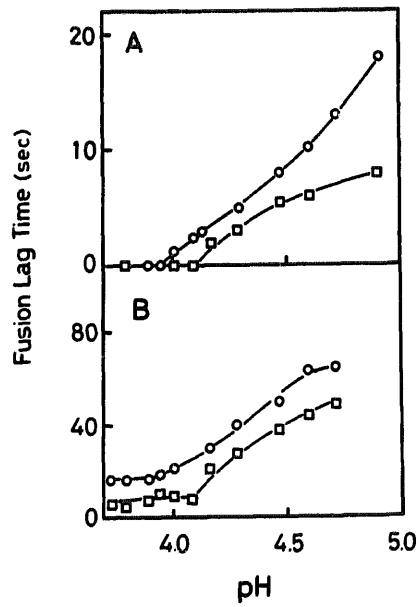


Fig. 5. pH dependencies of the fusion lag time at 25°C (A) and 10°C (B). The lag time was estimated from the intersect of the tangent of the maximum slope in the plot of fluorescence dequenching against time (Fig. 1). PS/PC(2:1) (○) and PS (□) liposomes were used.

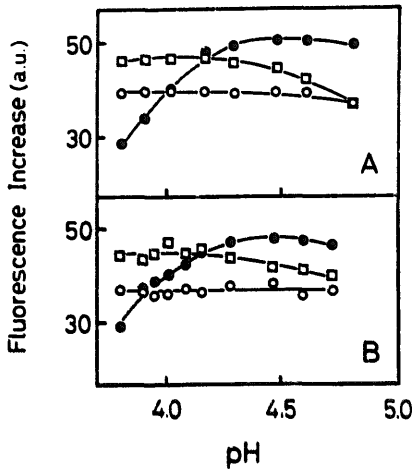


Fig. 6. pH dependencies of the increase in fluorescence of sensitized DACM measured at 25°C (A) and 10°C (B). Experiments were performed using mixtures of BIPM-clathrin and DACM-clathrin in the presence of PS/PC(2:1) (○) and PS LUV (□) and in the absence of liposomes (●) as described for Fig. 3. The fluorescence intensity at 470 nm at a steady-state level was measured.

clathrin might be related to the event(s) in the fusion lag-phase.

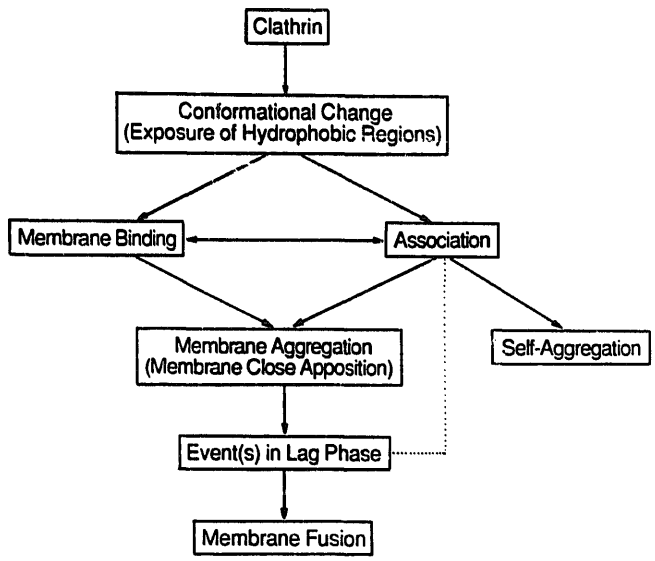
To clarify this relationship, we examined the pH-dependencies of the fusion lag-time and clathrin association in the presence and absence of liposomes in more detail. Fig. 5 shows the pH-dependencies of the fusion lag-times of PS/PC(2:1) and PS liposomes at 25 and 10°C. The lag time increased with increase in pH and decrease in temperature, as seen in Fig. 1. Interestingly, pH-thresholds were observed, and the threshold pH values for PS/PC(2:1) liposomes were about pH 3.9 and 4.1, respectively, at both 25 and 10°C. Fig. 6 shows the pH-dependencies of the extents of RET as indicators of clathrin association in the presence and absence of liposomes. The extent of RET increased with increase in pH in the absence of liposomes, but did not change markedly in the presence of PS/PC(2:1) or PS liposomes. Moreover, the curves in the absence of liposomes intersected those in the presence of PS/PC(2:1) and PS liposomes, and the pH at the crossover points for PS/PC and PS liposomes were about pH 4.0 and 4.2 at 25°C and about pH 3.9 and 4.2 at 10°C, which were similar to the threshold-pH values of the fusion lag-times. Thus, these results suggest that the final associated state of clathrin molecules on the liposome membranes may be related to the event(s) in the lag phase.

Discussion

To elucidate the general mechanism of membrane fusion induced by proteins at the molecular level, we have carried out a series of studies on fusion of phospholipid vesicles induced by clathrin [16–18]. In the present study, we determined the sequence of events in the fusion process.

First, we found that conformational change of clathrin occurs before membrane binding and association of clathrin. Next, we found that membrane binding and association of clathrin occur independently and simultaneously. We also found that in the absence of liposomes, several clathrin molecules assemble to form small soluble aggregates and then large insoluble aggregates. The latter two findings indicate that clathrin molecules bind to membranes and then associate with each other, or *vice versa*. Indeed, the initial rate of membrane binding of clathrin was higher than that of association of clathrin in the absence of liposomes below about pH 4.2, but lower than the latter above pH 4.2 (Fig. 4). The initial rates of liposome aggregation were apparently lower than those of membrane binding and association of clathrin (Figs. 2 and 3), indicating that membrane aggregation occurs after membrane binding and association of clathrin. Moreover, it was suggested that membrane binding and association of clathrin and membrane aggregation are not responsible for the fusion lag phase, and that some other event(s) occurs during the lag time.

From these findings, we conclude that membrane fusion induced by clathrin occurs through the sequence shown in Scheme I. At acidic pH, clathrin first changes its conformation through exposure of its hydrophobic regions. Then clathrin molecules either bind to membranes or associate with each other: some clathrin molecules bind to membranes after association and others form assemblies or clusters on membranes after binding. Subsequently, membrane aggregation is induced, and after an event(s) in the lag phase, actual fusion, that is, lipid mixing occurs. In the absence of



Scheme I. Possible process of clathrin-induced membrane fusion. The dotted line indicates that the associated state of clathrin is related to an event(s) in lag phase.

liposomes, clathrin molecules change in conformation and assemble to form large insoluble self-aggregates.

On the other hand, the threshold pH values in the pH-profiles of the fusion lag-time (Fig. 5) were similar to the critical pH values at which the final associated states of clathrin molecules were apparently reversed in the presence and absence of liposomes (Fig. 6), suggesting that this final associated state of clathrin on the liposome membranes may be related to the event(s) in the lag phase. Probably the interaction between clathrin molecules depends on pH more strongly in solution (in the absence of liposomes) than on liposome membranes (in the presence of liposomes), and the difference between this protein–protein interaction in these two states may affect the extent of protein–lipid interaction when the clathrin molecules are on the liposome membranes. This may explain the existence of a lag phase: in the pH region where the protein–lipid interaction would be stronger, the fusion lag-time would be zero or short, whereas in the critical pH region where the interaction would be weaker, the lag time would increase. In other words, an event(s) occuring in the fusion lag-time could be regulated by the balance between protein–protein and protein–lipid interaction on the membranes.

Lag phases have been observed in other fusion systems, such as the fusion reactions between 3T3 fibroblasts expressing hemagglutinin and erythrocytes [27] and between Sendai virus and erythrocytes [28]. In the latter case, the lag phase was suggested to be related to cluster formation and molecular rearrangement of viral proteins on the membranes. On the other hand, Blumenthal [29] proposed a model for membrane fusion induced by viral glycoproteins in which the proteins are arranged as oligomers, and these oligomers undergo a concerted conformational change in the fusion process. Using this model, Clague et al. [30] interpreted the lag time observed in the process of fusion between hemagglutinin-expressing cells and red blood cells in terms of the time required for multi-state transition from a fusion-inactive to a fusion-active state. Our results together with these reports suggest that the molecular rearrangement or conformational transition of proteins through interaction with surrounding lipid molecules may be involved in the event(s) in the fusion lag.

Previously, we have found that the terminal domain of clathrin has no ability to induce membrane fusion, but that the residual proximal part of the protein has fusogenicity [31]. Probably, clathrin molecules change in conformation with exposure of hydrophobic regions, possibly located in the proximal portion of the molecules, and bind to liposome membranes, which results in close apposition of the membranes. As a result, clathrin molecules in the assemblies or clusters could be rearranged between the two adjacent membranes, and lipid molecules in the regions of contact of membranes could be concentrated on the hydrophobic regions of clathrin molecules, which seems to be events in the lag phase. Further studies should clarify the actual event(s) in the lag phase of fusion, that is, the molecular structure of the intermediates triggering actual fusion, which is created by oligomeric forms of clathrin and lipid molecules after membrane aggregation.

In the current model for clathrin-coated vesicles, clathrin molecules interact directly with clathrin-associated proteins (adaptins), but not with membrane lipids [32], and in clathrin-liposome systems, the pH for induction of membrane fusion by the protein is lower than the cytoplasmic pH. It is thus unlikely that the fusion activity of clathrin is expressed in vivo. Consequently, we have used clathrin only to elucidate the general mechanism of protein-induced membrane fusion. However, if clathrin molecules released from coated vesicles encounter specific conditions that change their conformation to bind the proteins to the vesicle membranes in cells, they might express their potential fusogenicity in the process of endosome formation and endosome-lysosome fusion.

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