Conformation and the Properties of Porcine Brain Calmodulin Incorporated in Liposomes

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ABSTRACT: The conformation and properties of porcine brain calmodulin (CaM) in egg lecithin liposome were investigated by measuring fluorescence and circular dichroism (CD). Two to five percent CaM could be incorporated into the unilamellar vesicles and the fluorescence emission maximum of dancylated CaM was found to shift toward shorter wavelength by 20 nm. In contrast, a capture efficiency of CaM in multilamellar vesicles was 10 to 30% and the α -helical content of CaM increased with increasing lecithin concentration. In the absence of Ca^{2+} at the lecithin concentration of $5\text{--}10\text{ mg ml}^{-1}$, the CD spectrum suggested the same conformation for CaM which bound Ca^{2+} . In the presence of Ca^{2+} at $1\text{--}2\text{ mg ml}^{-1}$ lecithin concentration, the CD spectrum indicated the destruction of α -helical conformation, whereas, at $5\text{--}10\text{ mg ml}^{-1}$ lecithin concentration, the CD spectrum was the same with that of CaM upon calcium binding. The results of CD measurements were supported by fluorescence spectra.

KEY WORDS CD / Fluorescence Spectra / Liposome / Porcine Brain Calmodulin / Conformation /

Recently, liposomes have been widely used as models of biomembranes, because these are small vesicles made from lipid lamellae in which ions and low molecular weight materials can be trapped depending on the size of water pools of liposomes. Furthermore, liposomes can encapsulate high molecular weight materials such as enzymes, and proteins and incorporation of lysozyme and serum albumin into liposomes was successfully reported.^{1,2} Therefore liposomes are considered to work as a carriers for such materials incorporated.^{3,4}

However there are few studies on the nature

of high molecular weight materials incorporated into liposomes such as their conformation and biological functions, although the dynamic properties of membranes of liposomes have been actively studied by NMR and ESR.^{5,6}

In this study, conformational studies of CaM, which is one of the Ca^{2+} receptors and is incorporated into liposomes made from egg phosphatidyl choline, were carried out. CaM is adequate for studying the conformational change between the aqueous media and liposomes, since its primary higher structure and Ca^{2+} -binding site have been fully elucidated.⁷

Abbreviations: CaM, calmodulin; PIPES, piperazine-*N,N'*-bis(2-ethanesulfonic acid).

CaM was incorporated into unilamellar vesicles produced by ethanol injection method⁸ and reverse-phase evaporation technique (REV),⁹ and the results were compared with those in multilamellar vesicles.

EXPERIMENTAL

Materials

All chemicals were of high grade commercial products. CaM was isolated from porcine brain as previously described¹⁰ and dancylated according to the method of Johnson and Wittenauer.¹¹ Egg lecithin was obtained from Asahikasei Co., Ltd.

Preparation of CaM Incorporated in Lecithin Liposomes

In all the experiments, CaM was dissolved into 10 mM PIPES-KOH buffer so as to be incorporated into liposomes. The preparations of liposomes are briefly described below.

(1) *Unilamellar vesicles*: unilamellar vesicles were prepared by either of the following two methods.

(a) *Ethanol injection method*.⁸ Two volumes of egg lecithin dissolved in ethanol were added at the rate of 0.2 ml min⁻¹ into one volume of 10 mM PIPES-KOH buffer (pH 7.0).

(b) *REV method*.⁹ Three volumes of egg lecithin dissolved into diethyl ether were mixed with one volume of 10 mM PIPES-KOH buffer (pH 7.0), and the unilamellar vesicles were produced by evaporating diethyl ether after sonication for 10 min at 23°C under N₂.

(2) *Multilamellar vesicles*: Multilamellar vesicles were prepared by 10 min sonication at 23°C after adding 10 mM PIPES-KOH buffer (pH 7.0) containing CaM to egg lecithin dissolved in chloroform.

Fluorescence Measurements

In the present study, dancylated CaM was used throughout the experiments. Fluorescence emission spectra were measured with a Hitachi 650-10S spectrofluorometer.

The emission spectra of dancylated CaM were recorded at an excitation wavelength of 340 nm, and the spectra were corrected against the background spectra. The fluorescence intensity was the relative one against the intensity of dancylated CaM in an aqueous buffer.

Circular Dichroism (CD) Measurements

CD was measured with a JASCO J-40A automatic recording dichrograph at 23°C. The cylindrical cells of 0.2 mm path length was used. The solvent used was 10 mM PIPES-KOH, pH 7.0.

Gel Filtration Chromatography

Chromatography of liposomes was carried out on Sepharose 4B which was equilibrated and developed with 10 mM PIPES-KOH (pH 7.0) buffer. The absorbance at 410 nm and the fluorescence intensity at 490 nm excited at 340 nm were used to detect liposomes and dancylated CaM, respectively, in the elution.

RESULTS AND DISCUSSION

Gel Filtration Chromatography of Liposomes

Figure 1 shows the elution profiles of liposomes containing CaM in their inner aqueous phase on Sepharose 4B column.¹² In unilamellar liposomes prepared by ethanol injection method (Figure 1(a)), the elution peak for liposome did not coincide with that for CaM, but in the case of unilamellar liposomes prepared by REV method (Figure 1(b)), those two peaks eluted at the same position although the protein peak was small, indicating that some CaM molecules were incorporated into the unilamellar liposomes. The elution profile of multilamellar liposomes also showed fairly large peaks for liposomes and CaM at the same position, indicating that the capture efficiency of CaM was relatively high.

In order to evaluate the amount of CaM in multilamellar liposomes, free CaM molecules were expelled from liposomes suspensions by

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Figure 1. Gel filtration of liposomes on sepharos 4B. (a) Ethanol injection liposome. (b) Reverse-phase evaporation liposome. (c) Multilamellar liposome. (—), lipid determined by absorbance at 410 nm; (-----), CaM_{DANS} measured at 510 nm.

Figure 2. Fluorescence emission spectra of CaM_{DANS} in ethanol injection liposomes (a) and in multilamellar liposomes (b): ($\blacktriangle$), CaM in liposome in the presence of Ca^{2+} ; ($\square$), CaM in liposome in the absence of Ca^{2+} ; ($\bullet$), CaM in buffer solution (10 mM PIPES-KOH pH 7.8).

centrifugation at $100,000 \times g$ for 20 min. The CaM concentration in a supernatant solution was determined by the method of Bradford *et al.*¹³ By subtracting the amount of CaM in the supernatant from the initial amount of CaM in PIPES-KOH (pH 7.0) buffer, the capture efficiency was found to be about 30%.

Fluorescence Spectra of Dancylated CaM in Liposomes

Figure 2(a) shows fluorescence spectra of dancylated CaM in unilamellar liposomes prepared by ethanol injection method. The fluorescence maximum of dancylated CaM in aqueous media was observed at 510 nm, whereas it shifted to 490 nm in unilamellar liposomes. The fluorescence intensities of dancylated CaM in unilamellar liposomes also increased by 27% in the absence of Ca^{2+} and by 42% in its presence compared with the intensity in buffer solutions. In contrast, the fluorescence intensity of dancylated-CaM in a aqueous solution increased by 40% on addition of Ca^{2+} . The fluorescence maximum of dancylated CaM in the multilamellar liposomes was also blue-shifted to 490 nm, and the fluorescence intensity increased by 50% irrespective of the presence of Ca^{2+} (Figure 2(b)).

Figure 3. Effect of Mg^{2+} and Ca^{2+} on CaM fluorescence in multilamellar liposome.

When CaM was added to lecithin liposomes prepared by adding CaM to lecithin-chloroform solution before the addition of the buffer, blue-shift and enhancement of the fluorescence spectra were observed.

The effect of the concentrations of Ca^{2+} and Mg^{2+} on dancylated-CaM fluorescence using

Figure 4. CD spectra of CaM. (a) In ethanol injection and reverse-phase evaporation liposomes: (▲), CaM in ethanol injection liposome in the absence of Ca^{2+} ; (△), in the presence of Ca^{2+} ; (●), CaM in reverse-phase evaporation liposomes in the absence of Ca^{2+} ; (○), in the presence of Ca^{2+} . (b) Effect of multilamellar liposome in the absence of Ca^{2+} : (▲), [lecithin] = 1 mg ml⁻¹; (△), 2; (●), 5; (○), 10. (c) Effect of multilamellar liposome in the presence of Ca^{2+} : (▲), [lecithin] = 1 mg ml⁻¹; (△), 2; (●), 5; (○), 10.

by multilamellar liposomes was studied (Figure 3). With increase of Mg^{2+} concentration, fluorescence intensity decreased gradually, and a noticeable difference was not observed with or without the liposomes. In contrast, over 10^{-5} M Ca^{2+} , a remarkable decrease in fluorescence intensity was observed in the presence of liposomes. These results suggest that Ca^{2+} binds with CaM in unilamellar liposomes. On the other hand, it binds with a liposome membrane rather than with CaM in multilamellar liposomes, causing the decrease of membraneous fluidity and consequently the decrease of hydrophobicity in environment around CaM.

Circular Dichroism Spectra of Liposomes

CD spectra of the unilamellar liposomes containing CaM prepared by both methods of the ethanol injection and REV were shown in Figure 4(a). In the absence of Ca^{2+} , both liposomes showed almost the same values of $[\theta]_{220}$ as that for CaM in aqueous solutions. In liposomes by REV method, the value of $[\theta]_{220}$ gave $-14,000$, indicating the α -helix content was about 35% and was a little higher than

31% in liposomes prepared by the ethanol injection method. This difference seems closely relate to the differences in the size of liposomes and the capability to get incorporate the aqueous solution into the liposomes. In general, the diameter of the liposomes prepared by REV is in the range of 200 to 1000 nm, which is much larger than those prepared by the ethanol injection method, 30 to 110 nm. The REV method can provide liposomes with higher entrapping efficiency than the ethanol injection method. This high entrapping efficiency seems to decrease the rate of contact surface between CaM and lipid, and accordingly CaM in liposomes prepared REV method resembles more that in aqueous solution. In the presence of Ca^{2+} , the values of $[\theta]_{222}$ for CaM in liposomes prepared by the ethanol injection method and REV method were $-16,000$ and $-17,000$, respectively. These results suggest that on binding of Ca^{2+} , CaM molecules take on the α -helix enriched conformation.

The CD spectra of CaM incorporated into the multilamellar liposomes in the absence of Ca^{2+} were depicted in Figure 4(b) as a function of the concentration of the lipid. When

the lipid concentration was 1 mg ml^{-1} , the values of $[\theta]_{222}$ and $[\theta]_{208}$ were the same as those in the aqueous solution, indicating the α -helix content of CaM molecules was 31%. Figure 4(b) also shows that the magnitude of CD band increases as the lipid concentration increases from 2 to 5% although CD below 212 nm could not be measured because of turbidity increment, indicating that lipid interacts directly with CaM and makes the CaM more stable by increasing the α -helix content.

The same experiments were carried out in the presence of Ca^{2+} , and the results are shown in Figure 4(c). At relatively low concentrations of the lipids (1 and 2 mg ml^{-1}) the CD band at 222 nm was observed with the value of $-11,000$ to $-12,000$, but only the weak shoulder was observed at 210 nm. This suggests that the destruction of α -helical conformation and/or the increase of the β -structure of CaM molecules occurred by the interaction of Ca^{2+} -lipid with CaM. As the lipid concentration increases up to 10 mg ml^{-1} , the magnitude of CD band again increased and the values at 222 nm was $-17,000$, which was almost identical with that in the absence of Ca^{2+} and reveals that the α -helical conformation turns out to be predominant. The fact that the CD spectra of multilamellar liposomes were almost identical between in the absence and presence of Ca^{2+} indicates that Ca^{2+} interacts with lipid but not with CaM in multilamellar liposomes as stated above.

From above the results, the following conclusions were obtained.

1) The conformation of CaM molecules incorporated into the unilamellar liposomes can be easily changed by Ca^{2+} .

2) In the multilamellar liposomes, more CaM molecules were incorporated into liposomes than in unilamellar liposomes, and the conformation of CaM could be appreciably changed by increasing the lipid concentration.

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