Biochimica et Biophysica Acta, 1069 (1991) 229–234

BBAMEM 75401

Electrostatic interaction of poly(L-lysine) with dipalmitoylphosphatidic acid studied by X-ray diffraction

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(Received 28 February 1991)
(Revised manuscript received 16 July 1991)

Key words: Dipalmitoylphosphatidic acid; Poly(L-lysine); Electron density profile; Hydrocarbon chain packing; X-ray diffraction

Structure of dipalmitoylphosphatidic acid (DPPA) bilayers in the presence of poly(L-lysine) is proposed from the results of X-ray diffraction obtained by a storage phosphor detector with a high resolution called an imaging plate. The small-angle X-ray diffraction pattern exhibits that DPPA/poly(L-lysine) complex forms a highly ordered multilamellar structure. The electron density profile of the DPPA/poly(L-lysine) complex draws that only one poly(L-lysine) layer is intercalated between the neighboring DPPA bilayers. The wide-angle X-ray diffraction pattern suggests that the presence of poly(L-lysine) hardly affects the nature of hydrocarbon chain packing in the DPPA bilayers. The X-ray reflection from the DPPA/poly(L-lysine) complex indicates that the poly(L-lysine) molecules adopt a β-sheet conformation on the surface of the DPPA bilayers. The both surface areas occupied by a headgroup of the DPPA and by a lysine residue in poly(L-lysine) are estimated from the observed spacings. The number ratio of lysine residues to DPPA headgroups per unit area is greater than unity. Therefore, one DPPA headgroup interacts with more than one lysine residue electrostatically, i.e., the electric charge distributions in both the surface of a DPPA bilayer and the poly(L-lysine) β-sheet are incommensurate.

Introduction

Study on interaction between a polypeptide and an acidic phospholipid bilayer provides an insight into the association mechanism of extrinsic proteins with lipid bilayers and gives a clue to solve the structure vs. function relationship in biological membranes. Poly(L-lysine) has been used as a typical model peptide of extrinsic proteins, because poly(L-lysine) is expected to bind electrostatically to the surface of negatively charged lipid bilayers at physiological pH.

Poly(L-lysine) is known to affect both thermotropic properties and structures of acidic lipid bilayers; Addition of poly(L-lysine) to negatively charged lipid bilayers raises a gel to liquid-crystalline phase transition temperature [1–9]. In the complex of dipalmitoylphosphatidic acid (DPPA) and poly(L-lysine) the transition temperature increases from 50°C to 62°C at pH 9.0 [2–4], at which the DPPA headgroup might has two negative charges. On the other hand, in dimyristoylphophatidic acid (DMPA) the transition temperature increases by about 20°C at pH 7 [5,7]. But in the case of shorter poly(L-lysine) (< 4000 mol. wt.) the increase of transition temperature is much smaller [9]. Lateral phase separations induced by poly(L-lysine) have been observed in DPPA bilayers by electron spin resonance and fluorescence spectroscopy [2–4], and also in binary mixtures of DMPA and dimyristoylphospatidylcholine by Raman spectroscopy [9].

The conformation, which is given by the interaction between poly(L-lysine) molecules and acidic lipid bilayers, is of great interest to obtain fundamental knowledge in lipid–protein interaction. Structure of poly(L-lysine) molecules changes from random coil to ordered structure, when bound to negatively charged lipid bilayers, i.e., α-helical conformation on phosphatidylserine bilayers [10] and β-sheet structure on phosphatidic acid (PA) bilayers [9,11].

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Abbreviations: DPPA, dipalmitoylphosphatidic acid; DMPA, dimyristoylphosphatidic acid; PA, phosphatidic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EDTA, ethylenediaminetetraacetic acid; FWHM, full width at half-maximum; DSC, differential scanning calorimetry.

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Small-angle X-ray diffraction indicates that the DMPA/poly(L-lysine) complex forms a multilamellar structure [9] and Raman spectroscopy exhibits no significant change in the conformation of hydrocarbon chains of DMPA in the presence of poly(L-lysine) in the gel phase [9] However, the following points remain unclear; the number of β-sheets lying between the neighboring PA bilayers; the distance between adjacent polypeptide chains in a β-sheet; the packing of hydrocarbon chains of PA in the presence of poly(L-lysine). In the present paper, we will make clear the structure of the DPPA/poly(L-lysine) complex from the wide- and the small-angle X-ray diffraction patterns detected with a high-sensitive imaging plate.

Materials and Methods

Materials

Disodium salt of dipalmitoylphosphatidic acid (DPPA) was purchased from Avanti Polar Lipids, Inc. (Birmingham, AL, U.S.A.). Poly(L-lysine) hydrobromide of about 21500 mol. wt. was purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). All materials were used without further purification. 4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid (Hepes) and ethylenediaminetetraacetic acid (EDTA) were purchased from Katayama Chemical Ltd. (Osaka, Japan).

Sample preparation

A desired amount of DPPA was dispersed in a 200 mM Hepes buffer (pH 7.3) containing 5 mM EDTA. The dispersion after incubated at 80°C for 2 h was sonicated for 20 min, using a bath-type sonicator (Sine Sonic 100, Kokusai Denki, Tokyo, Japan). The lipid concentration was 200 mM. The complex of DPPA and poly(L-lysine) was prepared as follows. Poly(L-lysine) was dissolved in a Hepes buffer (pH 7.3). The poly(L-lysine) solution was added to the dispersion of the DPPA vesicles (200 mM). This solution is prepared so as to get a ratio of lysine residues/DPPA molecules of 2:1. The dispersion thus obtained was incubated at 90°C for 10 min and then cooled down to room temperature. This cycle of heating and cooling was repeated at least three times to get a homegeneous sample. The produced precipitates of the DPPA/poly(L-lysine) complex were collected by centrifugation at $9000 \times g$ for 15 min. As a result the concentration of DPPA used was 20–30 wt%. The samples were stored at 4°C for 4 days before the X-ray experiments.

X-ray diffraction

X-ray diffraction experiments were carried out at the station 15 A of the Photon Factory in National Laboratory for High Energy Physics (Tsukuba, Japan) [12]. The powder diffraction pattern was detected, using a storage phosphor detector called an imaging plate (Type BA-III, Fuji Photo Film Co. Ltd., Japan) [13]. Details of the experimental setup were described elsewhere [14]. The imaging plate is an integrating-type area detector which operates on the basis of laser-stimulated luminescence from a storage phosphor (BaFBr; Eu^{2+}) screen. It consists of a flexible plastic plate coated with 150 μm thick layer of phosphor polycrystals combined with organic binder. Even if the incident angle of X-ray is large on the plate, the spatial resolution on the imaging plate is scarcely lost since the traveling distance of X-ray beams crossing the phosphor part is almost unchanged. The size of the imaging plate is 185×185 mm. The dynamic range of the imaging plate is $1:10^5$, and the spatial resolution is 150 μm in full width at half-maximum (FWHM). The detective quantum efficiency of the imaging plate is bigger than 80% for X-ray. This value is 10–60 times as large as that in a usual X-ray film. The exposure time was less than 2 s in the present experiment. The distance from the sample to the imaging plate is about 20 cm. The spacings of diffractions were calibrated, using the (001) reflection (0.0998104 + 0.0000007 nm) of synthetic fluorophlogopite mica crystallites (National Bureau of Standards, Washington, DC, U.S.A.). The temperature was kept constant within ± 0.01°C by circulating water through the sample cell from a computer-controlled water bath (RCS-20D, Messgrerate-Lauda, F.R.G.). The background caused by the diffraction of water, polyimide film windows, etc. was subtracted. The two-dimensional X-ray diffraction data on the imaging plate was transformed into one-dimensional data as follows. First, the intensity with a particular diffraction angle was integrated by adding all the intensity data at the same distance from the center of the direct beam on the imaging plate. Second, the integrated intensity was divided by the number of data points at the same distance from the center. In such a way, we got one-dimensional X-ray diffraction pattern with a high signal-to-noise ratio. The data analysis was performed using FACOM M-780 at the Computation Center of Nagoya University.

Results

Small-angle X-ray diffraction of DPPA vesicles and of the DPPA/poly(L-lysine) complex

Using a synchrotron X-ray, the diffraction was observed with a high resolution and low background because of its high directivity and its intense beam. Fig. 1 shows the small-angle X-ray diffraction patterns for (A) pure DPPA vesicles and for (B) DPPA/poly(L-lysine) complex at 40°C in gel phase. There appears a broad reflection around 5.8 nm for pure DPPA vesicles (Fig. 1A). It is consistent with the fact that generally negatively charged lipid vesicles at low ionic strength

give rise to a broad scattering pattern in the small-angle region. The scattering pattern for pure DPPA vesicles obtained in this experiment is slightly sharper than the pattern for vesicles of phosphatidylserine [15] and cardiolipin [16]. This is due to the fact that the concentration of counter ions for DPPA contains at least 200 mM, because the DPPA used in this experiment is the disodium salt and furthermore counter ions are included in the Hepes buffer. For DPPA vesicles the FWHM of the small-angle reflection peak became narrow as the Na^+ concentration rises and finally at 1.2 M Na^+ a sharp reflection takes place at 5.78 nm (unpublished data).

In contrast to pure DPPA vesicles, the DPPA/poly(L-lysine) complex gives sharp reflections (Fig. 1B). Owing to the large correlation length, we can observe higher order reflections up to the 9th, while the 6th and 8th order reflections are weak and then undetectable (Fig. 1B). This result indicates that the complex forms a highly ordered multilamellar structure. The lamellar spacing of DPPA/poly(L-lysine) complex is determined to be 6.61 nm.

Wide-angle X-ray diffraction of DPPA vesicles and of the DPPA/poly(L-lysine) complex

The wide-angle X-ray diffraction patterns for both pure DPPA vesicles and for the DPPA/poly(L-lysine) complex at 40°C in gel phase are shown in Fig. 2. The spacing is related to lateral packing of hydrocarbon chains in phospholipid bilayers. There appears a single

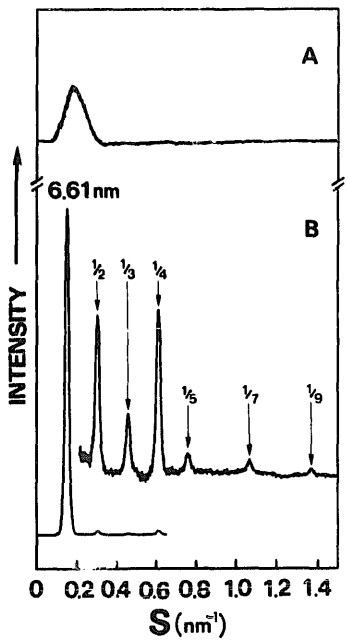

Fig. 1. X-ray diffraction patterns in the small-angle region for (A) DPPA bilayers and (B) DPPA/poly(L-lysine) complex at 40°C in gel phase, which were obtained from the data taken by imaging plates. The pattern of the 2nd to 9th order lamellar reflections for DPPA/poly(L-lysine) complex is inserted in an expanded scale in the ordinate.

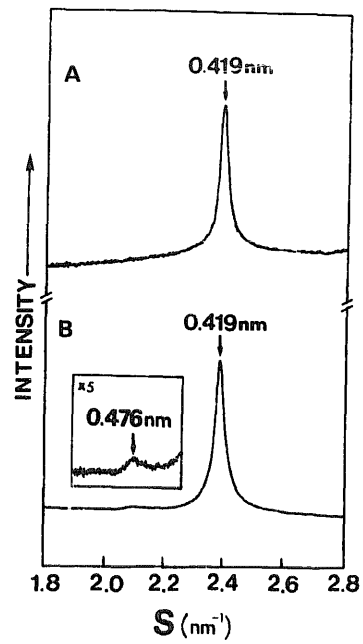

Fig. 2. X-ray diffraction patterns in the wide-angle region for (A) DPPA bilayers and (B) DPPA/poly(L-lysine) complex at 40°C in gel phase obtained by the same way as in Fig. 1. The inset shows an expanded version. A weak but fairly sharp reflection is clearly seen at 0.476 nm. This reflection corresponds to the spacing between the neighboring polypeptide chains in a β-sheet of poly(L-lysine).

sharp reflection at 0.419 nm in both pure DPPA vesicles and the DPPA/poly(L-lysine) complex. The spacing of hydrocarbon chains agrees with that in dihexadecylphosphatidic acid [17] and in phosphatidic acid (PA) [18]. The sharp and symmetric single peak indicates that the hydrocarbon chains are packed in a hexagonal lattice, which gives an area of 0.203 nm^2/chain. Essentially no difference appears in the spacing the shape in the 0.4 nm region between DPPA/poly(L-lysine) complex and pure DPPA vesicles. The fact suggests that the packing of hydrocarbon chains remains unaffected notwithstanding the addition of poly(L-lysine) in the gel phase.

Another conspicuous feature in the wide-angle diffraction pattern of the DPPA/poly(L-lysine) complex is that a weak but fairly sharp peak appears at 0.476 nm (Fig. 2B). This reflection is not explained in terms of any higher order lamellar diffractions of DPPA/poly(L-lysine) complex. Diffraction with periodicity between adjoining polypeptide chains in the β-sheet structure of homopolypeptide is expected to appear in the range between 0.45 and 0.49 nm. Actually, Suwalsky and Llanos [19] have observed the (200) reflection at about 0.47 nm for poly(L-lysine) hydrobromide crystal with β-sheet structure. On the other hand, it has been suggested by means of Raman spectroscopy [9] and circular dichroism [11] that PA vesicles induce a conformational change of the poly(L-lysine) molecule from a random coil to a β-sheet structure. Thus the

present X-ray diffraction result strongly suggests that the poly(L-lysine) adopts a β-sheet conformation when bound to DPPA bilayers. The appearance of the rather sharp diffraction at 0.476 nm indicates that fairly large ordered domains of β-sheet takes place on the surface of DPPA bilayers. It is briefly pointed out that the reflection at about 0.47 nm also appears in the DMPA/poly(L-lysine) complex. Therefore, the interaction between PA and poly(L-lysine) has immutable nature.

Electron density profile

Fig. 3 shows an electron density profile of the DPPA/poly(L-lysine) complex at 40°C in the gel phase with a resolution of 0.73 nm. The phase angle of each structure amplitude must be 0 or π for an one-dimensional electron density profile in a centersymmetric system. This is the case for lipid bilayers. Although generally phase angles are determined by performing a swelling experiment, this is not possible for the DPPA/poly(L-lysine) complex. The water concentration has no influence on the lamellar spacing of DPPA/poly(L-lysine) complex. Even for the sample dried under vacuum overnight the lamellar spacing hardly changed. Consequently, all combination sets of phases were systematically tested to determine the phase angle of each lamellar diffraction peak. Among the phase combinations of the first to the fifth order diffraction peaks, only one set of combinations give an electron density profile that explains a bilayer structure reasonably. The other set of phase combinations results in abnormal electron density profiles, such as large positive electron density peaks at the center of the bilayer. The adopted phases of the first to the fifth order diffractions (π, π, 0, π, π) are identical to those reported previously for dipalmitoylphosphatidylcholine in gel phase [20]. The phases for the diffraction orders of 7th and 9th (π, π) were determined so as to give the most uniform electron density distribution for the portion of hydrocarbon chains. This is a similar way to that made for dipalmitoylphosphatidylglycerol by Blourock and McIntosh [21]. The position with strong density in Fig. 3 corresponds to the DPPA headgroup and on the other hand, the trough with low density corresponds to the terminal methyl groups. The separation between the DPPA headgroups in the both surfaces of a bilayer is 4.93 nm, while the distance of headgroups between neighboring bilayers is 1.68 nm. It is worthwhile to point out that the latter distance is close to the intersheet distance of β-sheet in a three-dimensional poly(L-lysine) crystal, which varies from 1.62 to 1.69 nm depending on the relative humidity [19]. From the present result, we conclude that there exists only one β-sheet of poly(L-lysine) bridging the neighboring DPPA bilayers, and then, a highly ordered multilamellar structure is formed (Fig. 4).

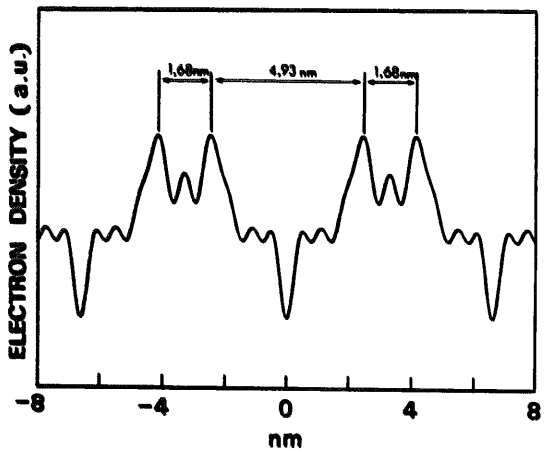

Fig. 3. A 0.73 nm resolution electron density profile for DPPA/poly(L-lysine) complex. In the profile, the highest density peaks correspond to the position of DPPA headgroups and the lowest density dips correspond to the position of terminal methylene groups appearing at the geometric center of a bilayer. The headgroup separation between both the surfaces of a bilayer is 4.93 nm. The space occupied by poly(L-lysine) lies between the adjacent highest density peaks, whose separation is 1.68 nm.

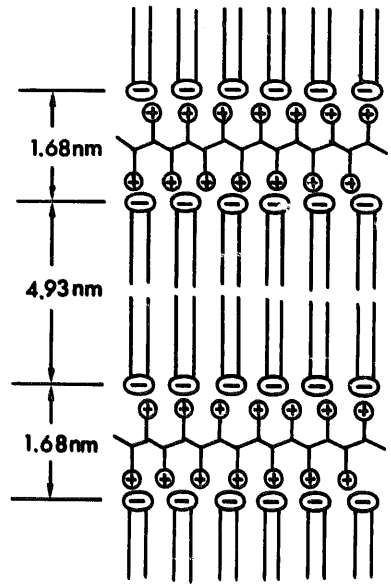

Fig. 4. Schematic representation of the DPPA/poly(L-lysine) complex. Poly(L-lysine) bridges the successively neighboring DPPA bilayer lamellae. Lysine residues arrange without specific sito-to-site interaction with DPPA headgroups.

Discussion

The X-ray diffraction study in the 0.4 nm region makes clear that the presence of poly(L-lysine) scarcely affects the nature of hydrocarbon chain packing. A poly(L-lysine) molecule shields the negatively charged surface of lipid bilayers and, however, has no specific site-to-site interaction with DPPA headgroups, There-

fore, like for Na^+ [22], the transition temperature might increase upon the addition of poly(L-lysine) as a consequence of an electric shielding effect. The shift of the transition temperature by the addition of poly(L-lysine) is greater than for Na^+ [5,9], probably because poly(L-lysine) provides effectively high ionic strength on the surface of the DPPA bilayers as expected structurally (see Fig. 4).

The electron density profile provides structural evidence that the complex forms a highly ordered multilamellar structure where only one β-sheet of poly(L-lysine) bridges the neighboring DPPA bilayers. This result suggests that DPPA/poly(L-lysine) complex forms a sandwich structure (Fig. 4). The formation mechanism of the multilamellar structure of DPPA/poly(L-lysine) complex is quite different from that of pure DPPA vesicles. A poly(L-lysine) molecule hardly permeates lipid bilayers because it is a kind of macromolecule with hydrophilic nature. In this study, poly(L-lysine) was added to the dispersion of DPPA vesicles (see Sample preparation). Therefore, in the course of the sandwich structure formation, not only local breakdown of the bilayers but also reconstruction of the multilamellar structure might happen.

Using an imaging plate, we successfully detected the X-ray weak reflection at 0.476 nm due to the structure of polypeptide bound to the lipid bilayer surface. This reflection was interpreted by the spacing between neighboring polypeptide chains in the β-sheet. The result reveals that the poly(L-lysine) forms a β-sheet structure on the surface of a DPPA bilayer. This is consistent with the fact that the spacing of 0.476 nm is very close to the spacing observed in a poly(-lysine) hydrobromide crystal [19]. The value of spacing obtained in the present experiment indicates that the hydrogen bond between polypeptide chains is not perturbed even when poly(L-lysine) forms a β-sheet structure on the surface of DPPA bilayers.

It is well known that there appear three distinct X-ray reflections in β-sheet crystals; the distance between neighboring β-sheets (0.5–1.6 nm) ruled by the species of residues, the distance between polypeptide chains in one sheet (0.46–0.49 nm) combined with hydrogen bonds, and the repeating distance of adjoining amino acid residues along one polypeptide chain. The theoretically estimated value of the last distance is 0.65 nm for parallel β-sheets and 0.70 nm for anti-parallel β-sheets [23]. In the present experiment, neither the reflection at 0.65 nm nor that at 0.75 nm was observed. This might be due to the fact that, in the DPPA/poly(L-lysine) complex, the poly(L-lysine) molecules take not only parallel but also anti-parallel β-sheet structures and both regions are not large enough to yield a clear X-ray reflection.

Let us compare the surface area of a DPPA headgroup with that of a lysine residue of poly(L-lysine) in the DPPA/poly(L-lysine) complex. The surface area occupied by a DPPA headgroup can be calculated from the observed spacing of hydrocarbon chain arrangement. The relationship between the cross-sectional area occupied by one hydrocarbon chain (S_{chain}) and the surface area occupied a headgroup (S_{head}) is approximately given by:

$$S_{head} = 2 \times S_{chain} / \cos \theta$$

where θ is the tilt angle of hydrocarbon chain to the bilayer normal. Before calculating the surface area occupied by a headgroup, it is necessary to determine the tilt angle. For this purpose, if the hydrocarbon chains are untilted, it is expected that separation between headgroups in the both sides of a bilayer for DPPA bilayers is about 4.9 nm in the framework of the CPK model. This is consistent with the headgroup separation 4.93 nm obtained from the present electron density profile (see Fig. 3). Furthermore, the wide-angle reflection shows only one sharp peak. These facts indicate that tilt angle is almost 0°. Therefore, the cross-sectional area occupied by one chain is easily estimated to be 0.203 nm^2. Then, the surface area occupied by a DPPA headgroup is about 0.4 nm^2. Secondly the area occupied by a lysine residue is calculated from the distances of β-sheet structure which has the spacing of 0.65–0.70 nm along the polypeptide chain as discussed above and 0.476 nm between the adjacent polypeptide chains. These distances give a surface area of 0.31–0.33 nm^2 per lysine residue of poly(L-lysine). As a result, the ratio of the number of lysine residues to DPPA headgroups per unit area is greater than unity.

This conclusion is supported by the results of differential scanning calorimetry (DSC) for DMPA/poly(L-lysine) complexes. When the number ratio of lysine residues to DMPA molecules is under about 1.25, both the transition peaks of pure DMPA (at about 50°C) and DMPA/poly(L-lysine) (at about 70°C) are observed and furthermore the transition enthalpy of pure DMPA decreases as the lysine/DMPA ratio rises. When the ratio is above about 1.25, the transition peak of pure DMPA disappears (unpublished data). These DSC results suggest indirectly that one PA molecule interacts with more than one lysine residue of poly(L-lysine) electrostatically. Thus, this might imply that the condition of electric charge neutralization does not hold locally, however, it might be the case that a part of the DPPA headgroups in a bilayer has two negative charges by dissociation and that consequently the electric charge neutralization condition is satisfied as a whole.

The present results conclude that the charge distributions make a mismatch between the surfaces of the DPPA bilayers and the β-sheets of the poly(L-lysine),

i.e., the periodicity of charge arrangements between them is incommensurate.

Acknowledgments

The authors are grateful to Dr. Y. Amemiya for helpful advice in the instrumentation of synchrotron X-ray diffraction experiments and Professor M. Akiyama for useful discussions. The authors wish to thank the Computation Center of Nagoya University for data analysis and the Photon Factory of National Laboratory for High Energy Physics. This work is supported in part by a Grant-in-Aid for General Scientific Research from the Ministry of Education, Science and Culture, Japan.

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