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The transition of α-helix to β-structure of poly(L-lysine) induced by phosphatidic acid vesicles and its kinetics at alkaline pH

Kohsuke Fukushima *, Takaki Sakamoto, Jun Tsuji, Keishi Kondo, Ryosuke Shimozawa

Department of Chemistry, Faculty of Science, Fukuoka University, Nanakuma, Fukuoka 814-01, Japan

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Abstract

Static and dynamic circular dichroism (CD) measurements were carried out for poly(L-lysine) in suspensions of dilauroylphosphatidic acid (DLPA) vesicles at alkaline pH (8–11.5). The static experiments demonstrated that the α-helix of poly(L-lysine) induced by deprotonation in alkaline solutions is transformed to β-structure by the addition of DLPA vesicles. Stopped-flow CD measurements for such order-to-order transition revealed that the rate determining step is the unfolding process of α-helix to random coil. Previously, we have reported the conformational change of poly(L-lysine) induced by DLPA vesicles at neutral pH, where the β-structure transition from random coil was observed. Thus two types of transition of poly(L-lysine) are observed depending on bulk pH, i.e., from random coil to β-structure and from α-helix to β-structure. So far the phospholipid-induced conformations of poly(L-lysine) were interpreted in terms of counterbalance between the positively charged terminals of the lysyl chains and the negative headgroups of the phospholipid in vesicle. However, present work indicates the direct interaction other than electrostatic interaction between the lysyl chain and phosphate groups of the lipid.

Key words: Poly(L-lysine); Alpha helix; Beta structure; Structure transition; Phosphatidic acid vesicle; Stopped-flow circular dichroism; Circular dichroism

1. Introduction

Phospholipid–protein interactions have been the major interest in relation to the biological and biophysical significance, since the picture of a biological cell had been drawn as the fluid phospholipid bilayers in which proteins were embedded [1–4]. Through various investigations, it is accepted that functional entities are proteins, and phospholipids provide the suitable environment controlling protein activity in addition to constructing the cell wall which separates cytoplasm from the surrounding.

Conformational changes of polypeptides upon interacting with phospholipid vesicles have been our interest, because the conformation is closely related to the functions of proteins. So far the pioneering work has been done on the conformational changes of polypeptides induced by phospholipids and/or surfactants [5–10]. In previous papers [11–13], we have investigated the conformational changes of poly(L-lysine) and lysine copolymers induced by acidic phospholipid vesicles at neutral pH. These experiments revealed that poly(L-lysine) undergoes a conformational change from random coil to β-structure upon interacting with dilauroylphosphatidic acid (DLPA) vesicles, where the driving force for inducing the β-structure was ascribed to the counterbalance between the positive charges of the lysine residues and the negative phospholipid headgroups.

Poly(L-lysine) is a basic polypeptide whose association constant for the terminal ϵ-amino group is reported as $pK_a = 10.0–10.3$ (at 25°C, KCl 0.1 M) [14,15]. Thus the charged state of lysyl chain changes from positive to neutral when the bulk pH exceeds its pK_a. This deprotonation results in α-helix conformation of poly(L-lysine). On the other hand, DLPA having a single net negative charge at neutral pH tends to gain an additional negative charge on exceeding pH about 8 [16]. These variations in the charged states of poly(L-lysine) and DLPA are expected to elucidate the pro-

* Corresponding author. Fax: +81 92 8656030.

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tein–phospholipid interactions in different environments.

The conformational change induced by phosphatidic acid vesicles at alkaline pH allows us, therefore, to obtain novel aspects for phospholipid–protein interaction. From this point of view, we measured circular dichroism (CD) spectra of poly(L-lysine) in the suspensions of DLPA vesicles and the mixed vesicle of DLPA/dilauroylphosphatidylcholine (DLPC) at various values of alkaline pH. Fortunately poly(L-lysine) shows well-known CD spectra which are characteristic for α-helix, β-structure, and random coil conformations depending on the solvent conditions. Using these reference spectra, the observed spectra in vesicle suspensions were analyzed with the least-squares curve fitting method to evaluate the fractions of the three conformations.

In addition to the static CD measurements, the kinetic characterization for the conformational transition of poly(L-lysine) is also carried out using time-resolved CD spectra obtained with stopped-flow CD apparatus.

2. Materials and methods

Materials

DLPA and DLPC were obtained from Sigma, and used without further purification. Vesicle suspensions were prepared as follows.

The weighed phospholipids were dissolved in minimum amounts of chloroform to give clear solution, following which the solvent was evaporated in vacuo. After adding buffer solution to the sample, the phospholipids were sonicated in a bath type sonicator under controlled temperature as reported before [13]. In the previous experiments [13], we adopted the extrusion method to have homogeneous single-compartment vesicles. In this experiment, however, we can not use an extruder because of the dissolution of the porous membrane in alkaline solutions. The concentration of the phospholipid in stock suspension thus prepared was about $1 \cdot 10^{-3}$ M. The vesicle size was monitored by a quasi-elastic light-scattering apparatus [17] at 35°C, and it was revealed that DLPA vesicles have hydrodynamic diameters varying from $1 \cdot 10^{2}$ nm at pH 8.0 to $3 \cdot 10^{2}$ nm at pH 11.5 with larger polydispersity in higher pH.

Poly(L-lysine) (Wako Pure Chemical, Mol. wt. 15000–30000) was dialyzed against 0.1 M HCl to convert hydrobromide to hydrochloride, and then against H_2O to remove excess HCl. The concentrations of the poly(L-lysine) solutions were estimated by the ninhydrin method after hydrolysis in 6 M HCl.

All samples were prepared using buffer solutions as follows; (1) 10 mM *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid (Tes) at pH 8.0, (2) 10 mM 3-cyclohexylaminopropanesulfonic acid (CAPS) at pH 10.0 and 11.0. For buuffer solutions at pH 11.5 (3), sample solutions were adjusted by adding NaOH, because the buffer solution prepared with CAPS is showing a considerable optical density around 190 nm above pH 11. In order to avoid pH change by air contamination, pH adjustments and preparation of samples were carried out under nitrogen atmosphere.

CD measurements

Samples for CD measurements were prepared by mixing the phospholipid suspensions with poly(L-lysine) to give appropriate concentration ratios, where the residue concentration of poly(L-lysine) was kept about $5 \cdot 10^{-4}$ M. CD measurements were carried out with a JASCO J-600 spectropolarimeter. The temperature was kept at 35°C otherwise noted.

Stopped-flow CD measurements were carried out with JASCO J-600 spectropolarimeter equipped with stopped-flow apparatus (SFC-5). Two solutions in the reservoirs thermostated at 10°C were rapidly introduced to a quartz cell of 10 mm path length after instantaneous mixing operated by nitrogen gas pressure. The mixing dead time and the dead volume for this apparatus were 2 ms and 70 μl, respectively. The mixing ratio for the apparatus was calibrated with a copper sulfate solution. The concentration of poly(L-lysine) after mixing was kept about $2 \cdot 10^{-4}$ M, and CAPS concentration was held as low as 1 mM to have a better signal-to-noise ratio at 208 nm at which stopped-flow measurements were carried out.

Poly(L-lysine) displays characteristic CD spectra for α-helix, β-structure, and random coil as shown in the inset in Fig. 1. These spectra were obtained under the following conditions: α-helix, NaOH solution (pH 11.5) at 4°C; β-structure, sodium dodecylsulfate (SDS) solution ($3.5 \cdot 10^{-2}$ M) at 35°C; random coil, buffer solution (pH 7.0) at 35°C. Using these reference spectra, the observed spectra in the phospholipid vesicles were analyzed by the least-squares curve fitting method after appropriate correction of the wavelength to cancel out the red shift of the spectra caused by multiple scattering in turbid samples [11].

3. Results

Static CD measurements

Fig. 1 shows the CD spectra of poly(L-lysine) in the absence and presence of DLPA vesicles at pH 8.0 together with the calculated spectra based on the best fit values using the reference spectra seen in the inset. CD spectral change indicates that the addition of DLPA vesicles induces the conformational change from random coil to β-structure in accordance with the

result observed at pH 7.0 [11]. This parallel result between pH 7.0 and pH 8.0 is reasonable because poly(L-lysine) has the same charged state at these pH values. The transition from random coil to β-structure has been well described in terms of the counterbalance between positively charged terminals of lysyl chains and negative headgroups of DLPA on the vesicle surface.

CD spectra obtained at pH 10.0 in the absence and in the presence of DLPA vesicle suspensions are seen in Fig. 2 together with the fraction of α-helix of poly(L-lysine) in solution as a function of bulk pH in the inset. As seen in the inset, poly(L-lysine) in random coil transforms into α-helix conformation above pH 10.0. The conformational transition induced by pH increase reflects a change for the charged state of the lysyl side chain from positive to neutral. This transition pH estimated from the CD titration curve is slightly lower than the reported value. This lower transition pH is attributed to the fact that poly(L-lysine) can adopt α-helix conformation even in not fully deprotonated state (the degree of dissociation < 0.3) [14]. Furthermore, the high temperature (35°C) employed in this experiment shifts the transition to lower pH values [14]. According to both the CD titration curve and the reported pK_a value, we conclude safely that poly(L-lysine) has no net charge above pH 10.0, at which poly(L-lysine) adopts complete α-helix conformation. In such alkaline pH, the addition of DLPA vesicles induces the conformational change from α-helix to β-structure.

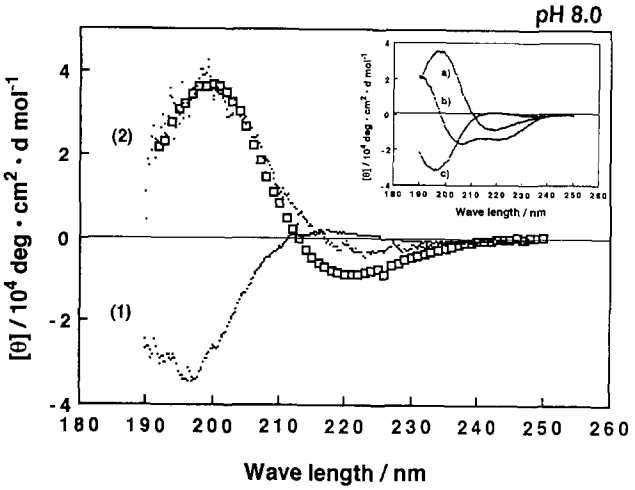

Fig. 1. CD spectra of poly(L-lysine) in buffer solution (1) and in DLPA suspension of $5.2 \cdot 10^{-4}$ M (2) at pH 8.0. □, indicates the calculated value estimated from the least-square method using the reference spectra in the inset. The fraction for the spectra (2) is estimated as 100% β-structure. (Inset) Reference CD spectra of poly(L-lysine); β-structure (a), α-helix (b), random coil (c). See text for detailed conditions.

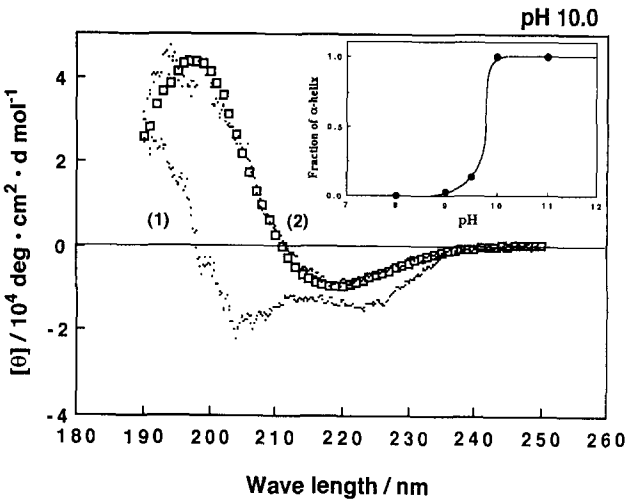

Fig. 2. CD spectra of poly(L-lysine) in buffer solution (1) and in the presence of DLPA suspension at $6.8 \cdot 10^{-4}$ M (2) at pH 10.0. □, indicates the calculated spectra of full β-structure. (Inset) The fraction of α-helix of poly(L-lysine) as a function of pH in bulk buffer solution.

The change in vesicle size caused by the introduction of poly(L-lysine) was monitored using the dynamic light-scattering method. At pH 10.0, the vesicle diameters increase from $2 \cdot 10^2$ nm without poly(L-lysine) to $3 \cdot 10^2$ nm at a mole ratio of DLPA to lysine residue $r = 1$. This increase in the hydrodynamic vesicle size indicates some aggregation of vesicles mediated by poly(L-lysine) in such a way that poly(L-lysine) in the β-structure on the vesicle surface bridges another vesicle.

Fig. 3a plots the estimated fractions of the three conformations at pH 8.0 as a function of DLPA concentration expressed as r, the mole ratio of DLPA to lysine residue of the polypeptide. The fraction of random coil decreases almost linearly with increase in DLPA concentration. Decrease in random coil conformation is compensated by increase in β-structure. Passing through the precipitation region, a further increase in r leads poly(L-lysine) in full β-structure. This profile is almost the same as the previous observation at pH 7.0, and is interpreted as stoichiometric binding of poly(L-lysine) to the vesicle surface by electrostatic interaction.

The estimated fractions at pH 10.0 are shown in Fig. 3b. The α-helix conformation of poly(L-lysine) linearly changes to the β-structure with increasing DLPA concentration accompanied by temporal appearance of random coil in the intermediate concentration range. Further increase in DLPA concentration induces complete β-structure passing through the precipitation region. It is interesting that poly(L-lysine) in α-helix is transformed into β-structure interacting with DLPA vesicles in spite of almost all poly(L-lysine) losing their

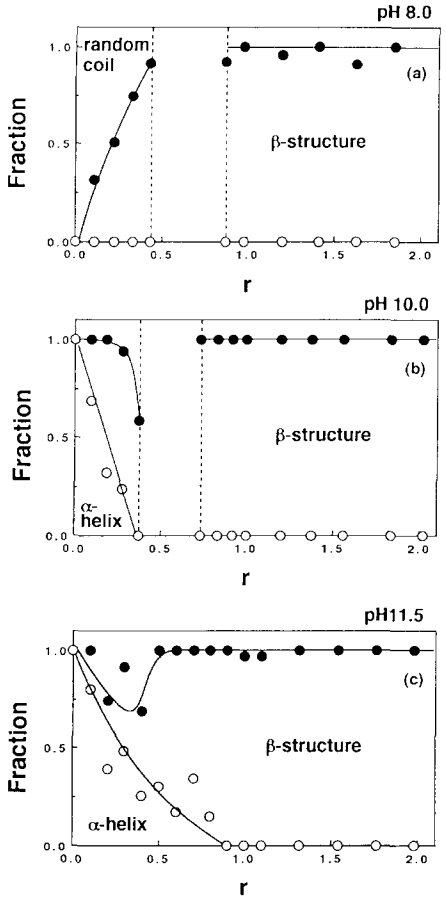

Fig. 3. Fraction profiles of poly(L-lysine) as a function of DLPA concentration expressed as r. pH 8.0 (a), pH 10.0 (b), pH 11.5 (c). Broken lines show the precipitation region.

positive charges at pH 10.0. So far transformation from random coil to β-structure has been interpreted in terms of counterbalance between positive lysyl terminals and negative headgroups of the lipid. How shall we describe the α-helix to β-structure transition taking place in the absence of the electrostatic driving force? Judging from the CD titration curve and the pK_a value, poly(L-lysine) is considered to be dissociated slightly even at pH 10.0. Thus, at this stage, we can not yet completely exclude the electrostatic interaction between the residual positive lysyl chain and doubly negative DLPA headgroups at this pH.

In order to ascertain whether poly(L-lysine) having neutral side chains can interact with DLPA vesicles, CD spectra were measured at pH 11.5. At pH 11.5, poly(L-lysine) side chain is reasonably considered to be neutral. In fact, CD spectra of poly(L-lysine) obtained at this pH have been regarded as the reference spectra for α-helix. In Fig. 3c, we can see that poly(L-lysine) in α-helix at pH 11.5 is transformed to β-structure on interacting with DLPA vesicles. Here at pH 11.5, α-helix to β-structure transition induced by DLPA vesicle is also confirmed. This indicates that we can not rely only on the electrostatic interaction to explain the α-helix to β-structure transition anymore because lysyl terminals of poly(L-lysine) at pH 11.5 are reasonably considered to be neutral through deprotonation. Hence, a driving force other than electrostatic interaction has to be taken into account.

At this stage, hydrophobic interaction is most promising to induce the β-structure at high alkaline pH, since a lysyl side chain has four methylene units in addition to terminal amino group. The hydrophobic region of DLPA vesicles is the bilayer of acyl chains with 12 hydrocarbons in each side. Thereby it is reasonable to recall the hydrophobic interaction between the lysyl side chains and acyl chains of the vesicle bilayer. At this point of view, we measured the CD spectra of poly(L-lysine) in the presence of DLPA/DLPC mixed vesicles at alkaline pH. A DLPC molecule with choline headgroup, which has no net charge at above pH 3, is suitable to elucidate the hydrophobic interaction between lysyl side chains and acyl chains of the vesicle bilayer. Before describing the results for the mixed vesicles, it is worth noting that pure DLPC vesicle induces no conformational change for poly(L-lysine) at neutral pH [11] and even at alkaline pH (data are not shown). This fact indicates that the driving force to induce the conformational change at alkaline pH is not ascribed to the hydrophobic interaction straightforward.

In Fig. 4, we plot the estimated fractions obtained for DLPA/DLPC mixed vesicle suspensions of mixing ratios 1:2 and 1:6 at pH 10.0. The concentration of

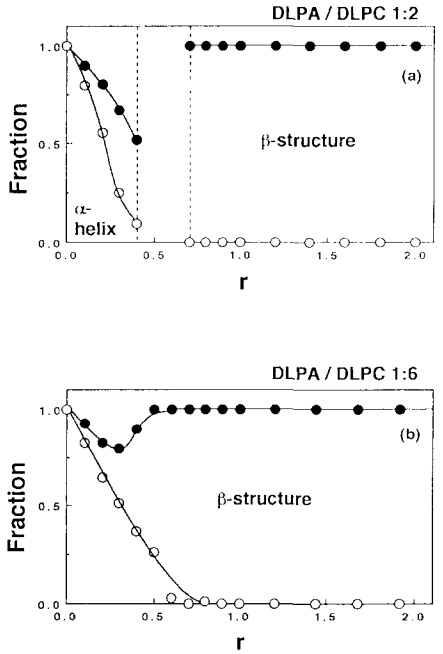

Fig. 4. Fraction profiles of poly(L-lysine) at pH 10.0 as a function of DLPA concentration expressed as r. The mixing ratios of DLPA/DLPC are 1:2 (a), 1:6 (b).

the phospholipid for the mixed vesicles is expressed as the mole ratio of DLPA to lysine residue of the polypeptide (r) instead of the total phospholipid concentration. At the mixing ratio of DLPA/DLPC 1:2, the fraction profile is essentially the same as that for pure DLPA vesicles. At the mixing ratio of 1:6, the fraction profile also indicates the transition from α-helix to β-structure at $r > 1$, and no precipitation is observed. This disappearance of precipitation by DLPC mixing takes place above the mixing ratio of 1:3. Precipitation has been interpreted in terms of the reduction of solubility of the peptide bound vesicles. In the mixed vesicle of the ratio exceeding 1:3, the mixed vesicles stably disperse even on binding of poly(L-lysine). Such stability in vesicle suspensions brought by DLPC mixing reminds us parallel phenomena that phosphatidylcholine stabilizes acidic phospholipid vesicles against divalent cation-mediated flocculations [18].

CD measurements were performed for various DLPA/DLPC mixing ratio up to 1:19. At any mixing ratios, the fraction profiles are subject to little effect of added DLPC on the induced conformation at $r > 1$; almost 100% β-structure is induced through pH 8.0–11.0 irrespective of mixing ratios. Because pure DLPC vesicles by themselves induce no conformational change of poly(L-lysine), it is remarkable that the mixed vesicles induce the β-structure even up to the mixing ratio of 1:19 at which the mole fraction of DLPA is as small as 0.05.

CD measurements for the same system were also carried out at 10°C in order to ascertain the contribution of the acyl chains to the induced conformation, since the phase transition temperature of DLPA has been reported to be 31°C [19]. If the β-structure of poly(L-lysine) was formed on inserting the lysyl chain into the hydrophobic acyl chains in the bilayer, the state of the bilayer (liquid-crystalline or gel) would affect the induced conformation. However, there are no significant differences between the fraction profiles at 35°C and those at 10°C, indicating that the β-structure is formed not putting lysyl chains deeply into the acyl chains but on the vesicle surface.

Stopped-flow CD measurements

In order to have kinetic aspects for the transition of α-helix to β-structure, stopped-flow CD measurements were carried out at pH 10 and 10.5. In general, the α-helix structure of a long chain polypeptide is more stable than that of short one. In particular, the transition kinetics from α-helix to other conformations would be subject to this effect; the longer the polypeptide, the lower the transition rate. This effect is divided into two factors, i.e., the initiation of cleavage at terminal residues and elongation of the successive cleavage. The former terminal effect is reduced with increasing chain length, and the latter is proportional to chain length.

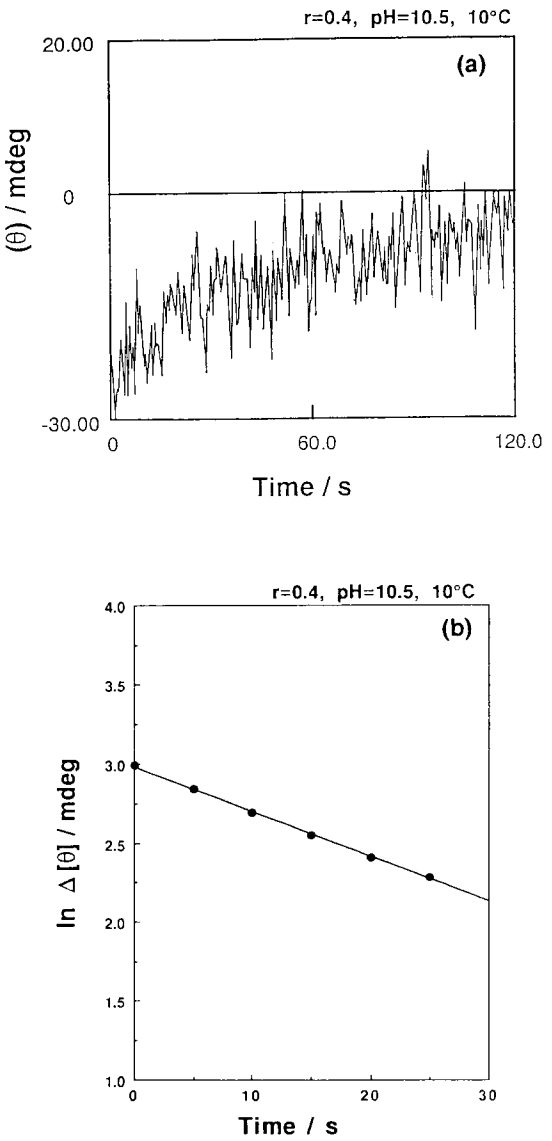

Fig. 5. Representative time-course of CD spectrum of poly(L-lysine) after mixing with DLPA vesicles at pH 10.5 (a). Semilogarithmic plot for ellipticity change with time (b).

At this stage, it is unclear which process is rate determining. Therefore, the obtained results should be understood as the average value for used poly(L-lysine).

As is seen from the reference spectra in the inset of Fig. 1, the difference in ellipticity between the spectra for α-helix and β-structure exhibits maximum around 208 nm. Hence, the ellipticity change on the transition was monitored at 208 nm. The typical time-course of ellipticity change at 208 nm is seen in Fig. 5a, where negative ellipticity for poly(L-lysine) positively increases after mixing with DLPA vesicles. This increase in ellipticity well corresponds to the transition from α-helix to β-structure observed in the static CD measurements.

The rate constant for the transition was evaluated by assuming the first-order kinetics. Semilogarithmic first order plot for the ellipticity in Fig. 5b gives linear

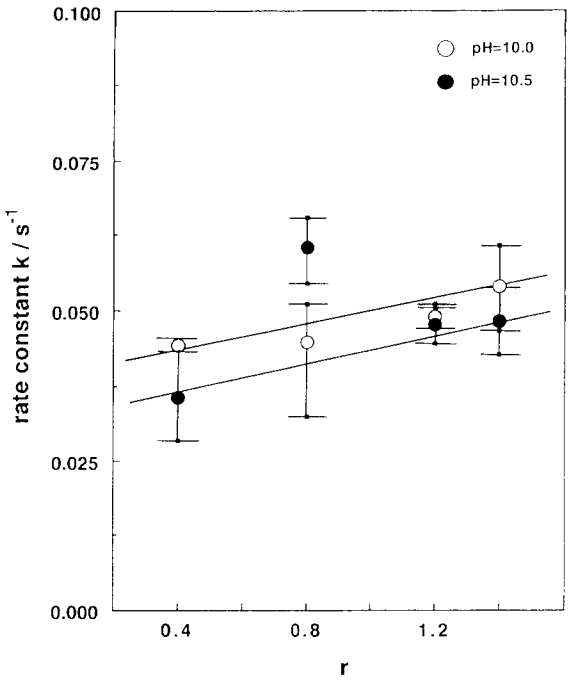

Fig. 6. The rate constants for α-helix to β-structure transition of poly(L-lysine) as a function of r at pH 10.0 and 10.5.

relationship between $[\theta]_\infty - [\theta]_t$ and time. The rate constant was evaluated from the slope of the plot, and is plotted as a function of DLPA concentration expressed as r (the mole ratio of DLPA to lysine residue) at pH 10 and 10.5 in Fig. 6, where error bars indicate the deviation of several runs. The rate constants thus obtained have values in the order of $1 \cdot 10^{-2}$ s^{-1}, and show a slight increase with r. The pH dependence of the rate constant is rather small. Rate constants for conformational changes have been reported for some polypeptides and enzymes [20–23]. Among these kinetic studies, rate constants vary two orders of magnitude from a fast process as coil to β-structure transition of poly(L-lysine) by sodium octylsulfate ($k \sim 1$ s^{-1}) to a slow process as conformational change of myoglobin by a sodium dodecylsulfate ($k \sim 1 \cdot 10^{-2}$ s^{-1}). The rate constants obtained in the present experiments indicate that vesicle induced α-helix to β-structure transition of poly(L-lysine) is a relatively slow process which is comparable to surfactant-induced α-helix to β-structure transition of poly(L-lysine) in unbufferd solution [20].

4. Discussion

In the present work, two major results were obtained from static CD measurements. (1) Poly(L-lysine) adopts the β-structure upon interacting with pure DLPA vesicles even in alkaline pH. (2) DLPA/DLPC mixed vesicles also induce the β-structure of poly(L-lysine) up to a mole fraction of DLPC as high as 0.95 in the mixed vesicle. So far disorder-order and the reversed transitions on polypeptide conformation induced by bioactive agents have been frequently observed, and have been interpreted in terms of the electrostatic and/or hydrophobic interaction between polypeptides and agents [22,24]. However, results obtained at alkaline pH can be explained by neither electrostatic nor hydrophobic interaction between poly(L-lysine) and DLPA. Hence we have to search for another concept to explain the results obtained here.

A specific interaction between deprotonated lysyl terminal hydrogens and DLPA phosphate oxygens seems to be responsible for the induced β-structure, since this region, which is so-called the hydrogen-bonding region, in-between polar and hydrophobic region has been left without consideration. From this point of

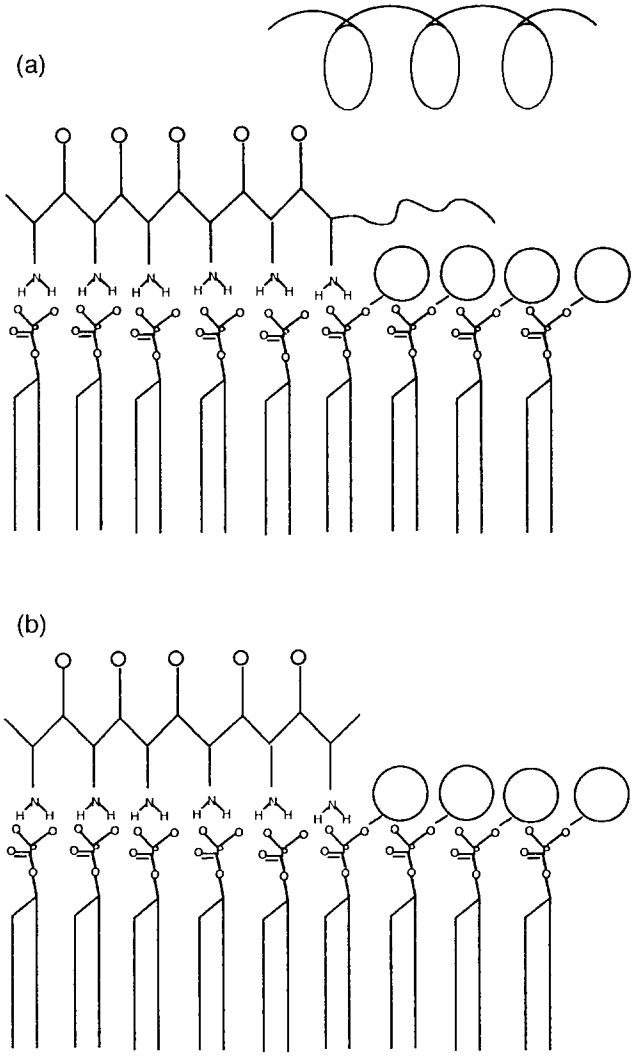

Fig. 7. Speculated schematic illustrations of β-structure induced by DLPA/DLPC mixed vesicle surface at low phospholipid concentration (a), and high phospholipid concentration as $r > 1$ (b). Relative sizes of each segment are based on the space filling model.

view, the fact that DLPC vesicles induce no conformational change on poly(L-lysine) is interpreted as the choline group attached to the phosphate group being so bulky as to prevent the lysyl chain approaching to the phosphate, i.e., smallness of the headgroup of phospholipid is a key word since the close approach is necessary to interact for hydrogen bonding.

Next, we have to answer why DLPA/DLPC mixed vesicles do induce the β-structure in alkaline solutions. This observation is in contrast to the results for the same system performed in neutral pH, where DLPC mixing leads to the reduction of the β-structure by as much as half that for DLPA/DLPC (1:1). Prior to discussing for the mixed vesicles, the definition of r for the mixed vesicles should be remembered; r is defined as mole ratio of DLPA to lysine residue of the polypeptide. Consequently, poly(L-lysine) would find enough binding sites of DLPA in the range of $r > 1$ even in the mixed vesicle. It is acceptable that in mixed vesicles, some sequential arrays of DLPA should be required so as to induce β-structure of poly(L-lysine). These sequential arrays of DLPA recall us a picture that DLPA rich regions and DLPC rich regions are induced by poly(L-lysine) in alkaline solutions. However, this does not imply complete phase separation between DLPA and DLPC but local heterogeneous distribution enough for β-structure formation in the mixed vesicles. Such local heterogeneous distributions induced by poly(L-lysine) have been reported for binary mixture of dimyristoylphosphatidic acid and dimyristoylphosphatidylcholine [25] as well as lateral phase separation of negative phospholipids/PC bilayers induced by external charges [26,27].

Speculated illustrations for the β-structure on the mixed vesicles are seen in Fig. 7 where poly(L-lysine) adopts the β-structure putting the lysyl terminal on (or between) the phosphate groups of DLPA in the mixed vesicle with the heterogeneous distribution of the phospholipids. Fig. 7a depicts the coexistence of the three conformations at low phospholipid concentration in which poly(L-lysine) takes the α-helix in bulk phase and the β-structure on the surface of DLPA molecules partly leaving in random coil away from the surface because of DLPC rich region. At high phospholipid concentration as $r > 1$ (Fig. 7b), poly(L-lysine) has enough binding sites on the heterogeneous mixed vesicle surface, and adopts the β-structure. This model explains the whole fraction profiles of poly(L-lysine) obtained at alkaline pH; the α-helix of poly(L-lysine) transforms into the β-structure with increasing phospholipid concentration, accompanied by temporal appearance of random coil in the intermediate concentration range.

The order-to-order transition has biological significance in concerned with an appearance and a disappearance of conformation-related biological function because the conformation, in particular ordered conformation, of a polypeptide closely relates to its function. Nevertheless, the kinetic aspects of polypeptides for the order-to-order transition like α-helix to β-structure transition have been limited to a few reports. The situation reflects the fact that a suitable condition to induce such a transition is rather difficult to find out. Fortunately DLPA vesicle-induced transition of poly(L-lysine) in alkaline buffer solutions is the case. Because this type of vesicle-induced transition takes place on the surface of the vesicle, the vesicle-induced transition is expected to offer a kinetic aspect, in particular, under two-dimensional restriction rather than usual the transition in a bulk phase.

In addition to the value of the rate constant itself, we are interested in the transition mechanism how such an ordered structure to be converted to another ordered structure. That is, the transition proceeds whether directly to final state or through an intermediate state, like random coil. Kinetic measurements reveal that the rate constants for the transition vary in the order of $1 \cdot 10^{-2}$ s^{-1}. This value of the rate constant is rather small compared with those for disorder-to-order transitions whose rate constants are in the order of 1 s^{-1}. A preliminary experiment performed on random coil to β-structure of poly(L-lysine) at neutral pH exhibited that the transition to the β-structure is too fast to be followed by the stopped-flow CD technique, employed in the present experiment. Conse-

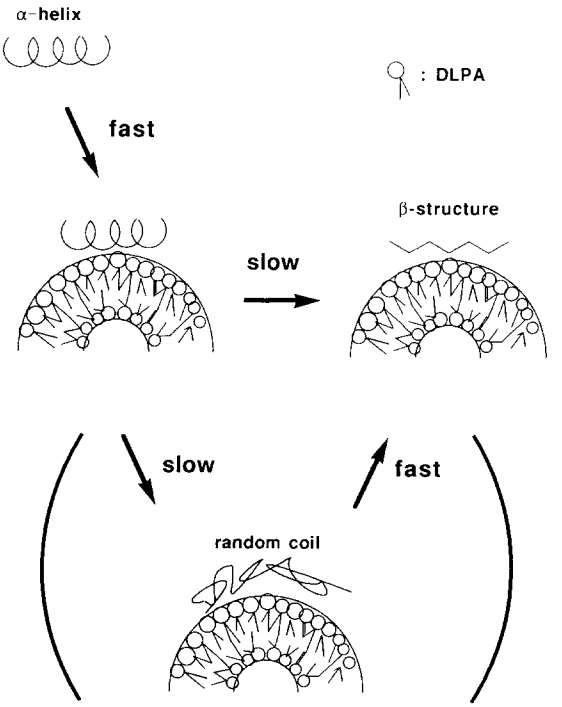

Fig. 8. Schematic illustration of the transition profile of poly(L-lysine) induced by DLPA vesicles at alkaline pH.

quently, the observed rate constants reflect relatively slow process other than random coil to β-structure transition. The most reasonable process determining the rate constant is the unfolding of the α-helix to a random coil, which process is followed by fast transition of random coil to β-structure.

The transition profile is illustrated in Fig. 8, where poly(L-lysine) in α-helix is bound on the surface of the vesicle at the first stage and then is distracted into random coil followed by fast reconstitution into β-structure. The rate constant for α-helix to β-structure transition of poly(L-lysine) induced by surfactant and 1-octanol has been reported to be $7.2 \cdot 10^{-2}$ s^{-1} at 20°C, and has been interpreted in such destruction-reconstitution mechanism [20]. Although these two transitions would correspond to the equivalent process, the rate constants for vesicle-induced transition are significant small even if we take account of lower temperature (10°C) operated in the present experiment. Slower kinetics observed for the present experiment may be responsible for the transition proceeding with two-dimensional restriction on the vesicle surface.

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