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BLOCK POLY(Ala)-POLY(Lys)

A WATER-SOLUBLE MODEL FOR INTRINSIC MEMBRANE PROTEINS?

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Summary

Block poly(Ala)$_{16}$-poly(Lys)$_{13.5}$ was synthesized by the Leuchs anhydride method. This polypeptide is water soluble in a largely monomeric form, but binds rapidly and spontaneously to unilamellar vesicles of dimyristoyl phosphatidylcholine at pH 7.4. The interaction is evidently of a hydrophobic nature since the complex is not disrupted by salt and no similar reaction is given by polylysine. Evidence for the interaction was obtained by ultrafiltration, chromatography on Sepharose 4B, and sedimentation velocity ultracentrifugation. While direct information on the molecular structure of the complex is still lacking, we propose that this amphipathic block copolymer binds to lipids in a similar manner as intrinsic membrane proteins and hence can be used to study the interactions of intrinsic proteins with lipids.

Introduction

Much of what we know about the fundamental properties of biological membranes has come from the study of model systems. The properties of membrane lipids have been investigated using lipid monolayers, planar bilayers, and vesicles. Detergent micelles, in turn, have been used as even simpler model systems which possess some of the thermodynamic characteristics of membranes [1]. More complex models for membranes include protein as well as lipid components. Several purified membrane proteins have been recombined with selected lipids to give model membranes having fewer components but similar functional characteristics to the membranes from which the proteins were derived. An effort has also been made in various laboratories to construct

membranes from lipids and synthetic polypeptides [2—8]. This endeavor has had limited success, however, due to the lack of suitable polypeptide models for intrinsic membrane proteins.

It is generally agreed that there are two major classes of membrane proteins [9,10]. The extrinsic proteins are understood to associate by predominantly (but not necessarily exclusively) electrostatic means with the surface of a lipid bilayer; whereas intrinsic proteins are those for which a portion of the protein penetrates into the interior of the lipid bilayer so as to give an appreciable degree of hydrophobic binding, but again, not necessarily to the exclusion of electrostatic or hydrogen-bonding interactions.

The interactions of basic polypeptides with acidic lipids probably resemble the bonding of some extrinsic proteins to membranes, and systems of this type have been widely studied [2—6]. Homopolymers of lysine and ornithine [3], and the positively charged random copolymers, poly(Glu, Lys, Ala), poly(Lys, Phe), poly(Lys, Tyr), and poly(Lys, Ser) [2—4] all readily bind to lipid vesicles containing acidic lipids. Weak interactions have also been found between poly(Lys, Phe), poly(Lys, Tyr), and poly(Lys, Ser) [3,4] with phosphatidylcholine, which is zwitterionic. Polylysine binds very weakly or not at all to phosphatidylcholine [2,7]. Poly(Glu), however, interacts with phosphatidylcholine vesicles as shown by NMR [7], glucose permeability and spin labeling [8]; the NMR data indicate that the principal interaction involves the lipid head groups.

Desirable features of a polypeptide model for intrinsic membrane proteins are water solubility, ability to bind hydrophobically to lipid, and availability of an analytical means for studying the interaction. We reasoned that since the amphipathic nature of intrinsic proteins appears to be their essential structural characteristic, we should be able to model this feature through the use of a block copolymer consisting of a string of polar amino acids followed by a string of non-polar amino acids. The polar portion should confer water solubility, and the non-polar portion ought to bind hydrophobically to lipid.

Ingwall et al. [11] found that sandwich copolymers of poly(Lys)-poly(Ala)-poly(Lys) are water soluble in a largely monodisperse form. They also found that the corresponding copolymers with poly(Lys) replaced by poly(Glu) are water soluble but occur in an aggregated form. We have therefore prepared block copolymers of Ala and Lys for testing as possible models of intrinsic membrane proteins [12].

A variety of methods are available for detecting and studying association reactions between lipids and proteins. Among these are spectroscopic methods, e.g. circular dichroism [2,3], fluorescence [13], NMR [7], and ESR spin labeling [8]. Analytical ultracentrifugation has also been used [14], as has density gradient ultracentrifugation [15], gel filtration chromatography [14], ultrafiltration [16], and thermometric titration [17,18].

We have used ultrafiltration, chromatography on Sepharose 4B, and analytical ultracentrifugation to characterize the interaction of poly(Ala)-poly(Lys) with unilamellar vesicles of dimyristoyl phosphatidylcholine. The results show that a strong hydrophobic binding of polypeptide to the lipid vesicles occurs spontaneously upon mixing, with the reaction being essentially complete within 15 min at room temperature. A concomitant aggregation or fusion of a portion of the vesicles evidently accompanies the binding. The potential signifi-

cance of this system for studying the interactions of lipids and intrinsic proteins has prompted us to communicate these results at the present time, even though the details of the interaction have not yet been fully explored.

Materials and Methods

Chemicals and solutions. Three polypeptides were used in the present work. Poly(L-lysine) was obtained from Sigma (type II). This polymer had a reported viscosity average molecular weight of 3400, corresponding to an average degree of polymerization of 26. The synthesis and characterization of poly(Ala)-poly(Lys) was previously described [12]. Briefly, the synthesis used the anhydrides of carbobenzoxy-L-lysine and carbobenzoxy-L-alanine. Cyclohexylamine was the initiator [11,12]. Sedimentation equilibrium ultracentrifugation of this polymer gave a weight average molecular weight of 2900. The plot of d(ln c) versus d(r^2) was linear over 95% of the cell, which indicates that the polymer was largely monodisperse, except for a small amount of rapidly sedimenting material. The molar ratio of Ala : Lys was 1 : 0.86, as determined by amino acid analysis. These data give an average chain composition of $(Ala)_{16}$-$(Lys)_{13.5}$.

The third polymer employed was poly(Lys)-poly(Ala)-naphthyl. Its synthesis and characterization were carried out in a similar manner as for poly(Ala)-poly(Lys) except that α-naphthylamine was used as the initiator, and poly(Ala) was C-terminal rather than N-terminal. The molecular weight of the resulting product was found to be 1200 as determined by sedimentation equilibrium. Amino acid analysis yielded an Ala : Lys ratio of 2 : 1, which give an average chain composition of $(Lys)_4$-$(Ala)_8$-naphthyl. This polymer was also water soluble and largely monodisperse as indicated by the ultracentrifugation results.

Dimyristoyl phosphatidylcholine (98% pure) was purchased from Sigma and used without further purification or analysis. Vesicles were prepared by sonication in a Branson sonifier (Model W-350) using an immersion tip. Sonication was carried out at 25—30°C until the solution was clear; this usually required a total sonication time of 30—45 min, using a duty cycle of 50% time on. The sonicated solution was passed through a Millipore filter (0.45 μm pore size) which effectively removed undispersed lipid as well as debris from the sonicator tip. The solutions were sonicated the same day as they were used. Phospholipid concentrations were determined by a modification of the method of Fiske and Subbarow [19].

All solutions of vesicles and polypeptides were prepared in 10 mM Tris-HCl buffer at pH 7.4 unless otherwise noted.

Ultrafiltration. An Amicon Model 52 ultrafiltration cell was employed, using a nitrogen pressure of 30 lb/inch^2; PM30 filters with a nominal pore size corresponding to 30 000 daltons were obtained from Amicon. These filters retain lipid vesicles, but all of the polypeptides used in this work were small enough to pass through. After each use the filters were rinsed with water and soaked for 15 min in 2 M NaCl. The filters were stored in deionized distilled water at 2°C.

The following procedure was used to study polypeptide-vesicle interaction by the ultrafiltration method. First, an aliquot from a stock solution of poly-

peptide (20 mg/ml) was diluted to 1 mg/ml. The CD spectrum of this solution was recorded, after which 10 ml of the diluted solution was applied to the ultrafilter and the CD spectrum of the first 2 ml of filtrate recorded again. The ratio of measured ellipticities at 225 nm gave the percent recovery of polypeptide in the absence of vesicles. This value varied from 62 to 93% (for the various polypeptides), and was used to normalize recoveries obtained in the presence of vesicles. Proper functioning of the filters was checked by carrying out a polypeptide filtration both before and after a filtration involving vesicles.

Mixing experiments of polypeptide and dimyristoyl phosphatidylcholine vesicles were performed at room temperature (22—23°C). Either a 1 : 1 or 23 : 1 weight ratio of polypeptide to lipid was employed. A typical experiment was carried out in the following manner. 1 ml each of stock solutions of polypeptide (20 mg/ml) and dimyristoyl phosphatidylcholine (20 mg/ml) were mixed, followed immediately by a ten fold dilution of half of the mixture to give a final concentration of 1 mg/ml for each component. The CD spectrum of the diluted mixture was recorded, and 9 ml of solution was promptly applied to the ultrafiltration cell. The CD spectrum of the first 2 ml of filtrate was again determined. The time lapse from initial mixing until the filtrate was collected was about 15 min. The percent recovery of polypeptide in the filtrate was computed from the ratio (filtrate/unfiltered) of ellipticities at 225 nm. This figure was in turn corrected for the partial retention of polypeptide alone by the filter in the absence of lipid, as described in Table I. (Ellipticities could be used as a relative measure of polypeptide concentration in the presence and absence of lipid since there were no detectable qualitative or quanitative changes in the CD spectrum upon the addition of lipid.) The CD spectra were recorded at room temperature using a JASCO ORD/UV-5 spectrophotometer, with a 1 mm or 10 mm cell path length.

Gel filtration chromatography. An 0.9 × 50 cm column of Sepharose 4B was packed and eluted with 0.03 M phosphate buffer, pH 7.0, in which the ionic strength was brought up to 0.2 M with KCl. An 0.5 ml sample of lipid vesicle/polypeptide mixture was applied to the column. All experiments were at room temperature. An ISCO UA-5 absorbance monitor operated at 254 nm was used to follow the elution profile. At this wavelength the apparent absorption is mainly due to light scattering by lipid vesicles, since the polypeptides employed in the column work lack chromophoric groups. The void volume and internal volume were determined using blue dextran and potassium dichromate, respectively. The apparent vesicle radii were calculated from the elution volumes using the error function analysis method of Ackers [20]; the calibration curve for Sepharose 4B of Brunner et al. [21] was adopted.

Ultracentrifugation. Sedimentation velocity ultracentrifugation was performed on a Beckman Model E analytical ultracentrifuge, using an AN-D rotor. The speed in all runs reported here was 48 000 rev./min, and the temperature was 25°C. A double-sector centerpiece was used in all experiments. The schlieren pattern was photographed every 4 or 8 min. Sedimentation coefficients were obtained by the second moment method [22], in which the area under the peak rather than the peak position itself is used in the calculation.

Results

Ultrafiltration

Table I summarizes the results obtained by ultrafiltration for the three polypeptides in the presence and absence of dimyristoyl phosphatidylcholine vesicles. Several points are worthy of note. First, the percent recovery of polylysine in the absence of vesicles is less than 100% under the conditions of the measurements. For this reason, 'corrected' recoveries as defined in the table are also given for the lipid/polypeptide mixtures. The corrected recovery of polylysine in the presence of lipid vesicles was about 70%, but addition of 0.15 M KCl to the polylysine and dimyristoyl phosphatidylcholine solutions either before or after mixing caused this value to increase to about 90%. It therefore appears that there was some interaction between polylysine and the lipid vesicles, but that this interaction is weakened by the addition of KCl. Since no effort was made to free the dimyristoyl phosphatidylcholine of possible acidic impurities, it is quite likely that the partial retention of polylysine in the presence of vesicles was due to electrostatic effects.

The recovery of poly(Ala)-poly(Lys) in the presence of lipid at a 1 : 1 weight ratio stands in sharp contrast to the recovery of polylysine. In this case, a corrected recovery of only about 10% was obtained. Since poly(Ala)-poly(Lys) is salted out of solution by 0.15 M KCl, it was not possible to study the interaction process in the presence of added salt. Addition of KCl after mixing the polypeptide with dimyristoyl phosphatidylcholine did not salt out the polypeptide and did not change the degree of recovery by ultrafiltration, however.

While we have not yet carried out a systematic study of retention as a function of the polypeptide to lipid ratio, one additional ratio was studied in which poly(Ala)-poly(Lys) was present in great excess (23 : 1 polypeptide to lipid

TABLE I

ULTRAFILTRATION OF POLYPEPTIDES IN THE PRESENCE AND ABSENCE OF DIMYRISTOYL PHOSPHATIDYLCHOLINE VESICLES

Solutions were prepared in 10 mM Tris buffer at pH 7.4; 0.15 M KCl was added as indicated. Actual percent recovery is defined as (polypeptide concentration in the initial filtrate fraction)/(polypeptide concentration in unfiltered material) × 100. S.D. (for more than two determinations) or the range (for two determinations) are given. Corrected percent recovery is defined as (actual percent recovery of lipid-polypeptide)/(actual percent recovery polypeptide alone) × 100.

Sample	Peptide/lipid (g/g)	No. of determinations	Percent recovery	
			Actual	Corrected
Polylysine	—	3	74 ± 6	
Polylysine + 0.15 M KCl	—	1	87	
Polylysine	1.0	2	50 ± 7	68
Polylysine + 0.15 M KCl	1.0	2	77 ± 1	88
Poly(Ala)-poly(Lys)	—	6	62 ± 7	
	1.1	2	4 ± 0.4	10
	23	4	48 ± 5	77
Poly(Lys)-poly(Ala)-naphthyl	—	6	93 ± 7	
	1.2	2	78 ± 3	84

weight ratio). The corrected recovery in this case was similar to that found for polylysine and dimyristoyl phosphatidylcholine.

The recovery values for the smaller block polymer, poly(Lys)-poly(Ala)-naphthyl, at a weight ratio of 1.2 : 1 polypeptide to lipid, were similar to those for polylysine in the presence of dimyristoyl phosphatidylcholine vesicles. It appears, therefore, that this polypeptide did not bind to the lipid vesicles to a significant degree.

Gel filtration chromatography

Additional evidence for the interaction of poly(Ala)-poly(Lys) with dimyristoyl phosphatidylcholine vesicles was obtained through the use of column chromatography on Sepharose 4B. Representative results are given in Fig. 1. The top section of Fig. 1 gives the elution profile of lipid vesicles; a particle radius of 110 Å was computed for the major peak [20,21]. In a column of this type, undispersed lipid or aggregated vesicles would be expected to elute in the void volume, at or near the solvent front. The absence of a peak at this position in Fig. 1 (top) indicates that the vesicle preparation is essentially free of such material.

Fig. 1 (middle) shows the result obtained when poly(Ala)-poly(Lys) was added to dimyristoyl phosphatidylcholine vesicles at a weight ratio of 1.3 : 1. A major central peak is again seen at the same position as for free lipid, but the

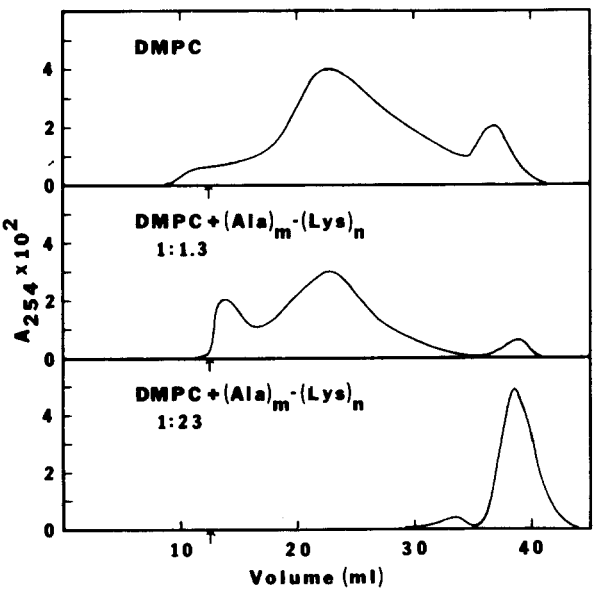

Fig. 1. Gel filtration on Sepharose 4B. An 0.9 × 50 cm column was eluted at room temperature with 30 mM phosphate buffer, pH 7.0, brought up to an ionic strength of 0.2 with KCl. Apparent absorbance (due mainly to light scattering) was monitored at 254 nm. Top. 0.5 ml of a 19 mg/ml suspension of dimyristoyl phosphatidylcholine (DMPC) vesicles. Middle. 1 ml of a solution containing 5.9 mg/ml poly-(Ala)-poly(Lys) and 4.6 mg/ml of dimyristoyl phosphatidylcholine (1.3 : 1, w/w). Bottom. 0.5 ml of a solution containing 10 mg/ml poly(Lys)-poly(Ala) and 0.44 mg/ml dimyristoyl phosphatidylcholine (23 : 1, w/w). The apparent absorption is not directly proportional to concentration of eluant. The arrows indicate the void volume peak location as determined with blue dextran.

minor peak close to the void volume suggests that a population of larger or aggregated particles is now also present. The similar elution pattern for the central peak in the presence and absence of polypeptide shows that the essential integrity of an appreciable fraction of the vesicles has been maintained.

The elution profile of a mixture of poly(Ala)-poly(Lys) with dimyristoyl

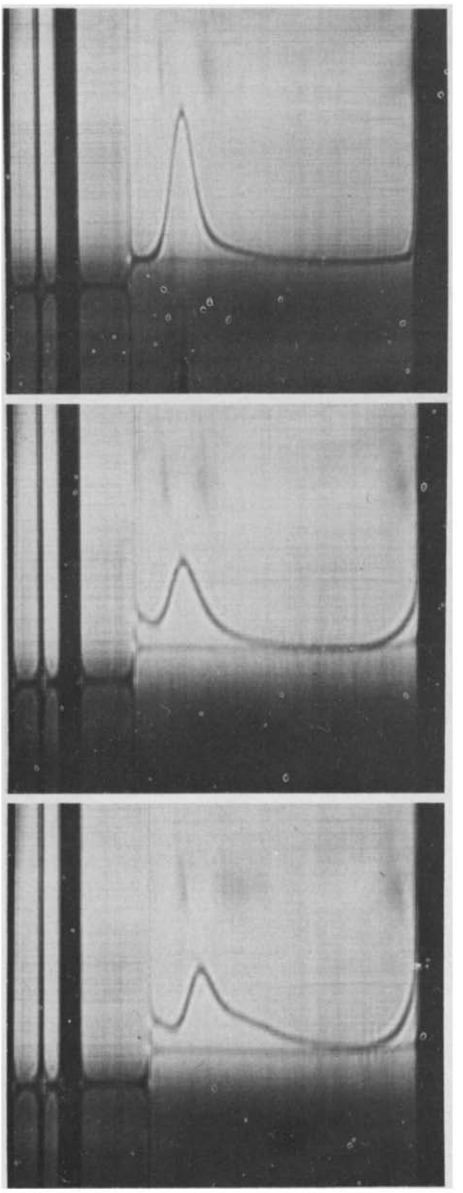

Fig. 2. Ultracentrifugation of vesicle/polypeptide mixture. All runs were carried out at 25°C, at 48 000 rev./min, in 10 mM Tris, pH 7.2. Top. Dimyristoyl phosphatidylcholine vesicles 19 mg/ml, at 42 min. Middle. Dimyristoyl phosphatidylcholine 10 mg/ml, and polysine 10 mg/ml, at 37 min. Bottom. Dimyristoyl phosphatidylcholine 10 mg/ml, and poly(Ala)-poly(Lys) 10 mg/ml at 37 min. The phase plate angle was the same for all plates.

TABLE II

SEDIMENTATION COEFFICIENTS OF DIMYRISTOYL PHOSPHATIDYLCHOLINE/POLYPEPTIDE MIXTURES

See legend to Fig. 2 for conditions of experiments. The s°_{25} values were extrapolated to infinite dilution, but were not corrected for the density of the buffer, DMPC, dimyristoyl phosphatidylcholine.

Components	s°_{25} (± 0.1)
DMPC	5.2 S
DMPC/polylysine (1 : 1 weight ratio)	5.2 S
DMPC/poly(Ala)-poly(Lys) (1 : 1 weight ratio)	8.2 S

phosphatidylcholine at a polypeptide to lipid weight ratio of 23 : 1 is shown in Fig. 1 (bottom). Virtually all of the material detected at 254 nm eluted with the internal volume. The great difference in elution profiles between the middle and bottom sections of Fig. 1 constitutes further proof that the polypeptide and lipid were associated when the ratio was 1.3 : 1 (middle, Fig. 1). The absence of a detectable lipid vesicle peak in the bottom section of Fig. 1 may mean that the lipid eluted with the polypeptide in the internal volume as a paucimolecular complex, but this is uncertain considering the small amount of lipid initially applied to the column.

Ultracentrifugation

The schlieren patterns for dimyristoyl phosphatidylcholine vesicles alone, dimyristoyl phosphatidylcholine plus polylysine, and dimyristoyl plus poly-(Ala)-poly(Lys) in a 1 : 1 mixture are given in Fig. 2. No schlieren peaks were seen for either of the polypeptides when centrifuged in the absence of lipid (not shown), which is in agreement with expectation under these conditions, considering their low molecular weights. The sedimentation coefficeints, extrapolated to infinite dilution, are given in Table II. The value of s^{0}_{25} for vesicles (5.2 S) is close to that of 5.33 S reported for dimyristoyl phosphatidylcholine vesicles by Watts et al. [23].

Addition of poly(Ala)-poly(Lys) to the lipid vesicles in a 1 : 1 weight ratio yielded a distinctly unsymmetrical schlieren peak (Fig. 2, bottom), and an increased sedimentation coefficient as determined by the second moment method [22] (Table II). By contrast, no change in sedimentation coefficient occurred when polylysine was added to the vesicles in a 1 : 1 weight ratio, and the schlieren peak remained symmetrical (Fig. 2, middle). These results are interpreted to mean that the block polymer interacted with the vesicles, but that polylysine did not interact to a detectable extent. The unsymmetrical schlieren peak indicates that the sedimenting material was heterogeneous with respect to size or density, or perhaps both of these.

Discussion

The evidence in hand demonstrates quite clearly that poly$(Ala)_{16}$-poly-$(Lys)_{13.5}$ interacts with vesicles of commercially obtained dimyristoyl phosphatidylcholine to a much greater degree than does poly$(Lys)_{26}$. Considering

the non-ionic, hydrophobic nature of poly(Ala), which by itself is water insoluble, it is reasonable to conclude that the binding of poly(Ala)-poly(Lys) to dimyristoyl phosphatidylcholine vesicles must be due mainly or solely to hydrophobic interactions involving the alanyl segment.

We do not yet have data which bears directly on the molecular structure of the lipid-polypeptide complex. The stoichiometry of the complex is also uncertain, considering the chromatographic and ultracentrifuge evidence for particle heterogeneity following interaction. The original reason for preparing poly(Lys)$_4$-poly(Ala)$_8$-naphthyl was to use the fluorescence of the naphthyl group as an indicator of the mode and degree of association, but its lack of interaction with lipid rendered this experiment impossible. We tentatively attribute its non-interaction to its short average chain length.

Our working hypothesis is that the alanyl segment of poly(Ala)-poly(Lys) penetrates into the lipid bilayer of the vesicles, and that the lysyl segment remains in the aqueous phase. Should this picture prove to be correct, then polymers of this type may indeed be used as tailor-made models for intrinsic membrane proteins, having simple and controllable compositions. The water solubility of poly(Ala)-poly(Lys) and its rapidity of interaction with lipid vesicles particularly recommends it for use in thermodynamic studies. The possible uses of block copolymers for modifying the properties of intact biological membranes, or even as carriers of pharmacological agents, remain to be explored.

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