Interaction of Type I Collagen Fibrils with Phospholipid Vesicles

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Abstract

Type I collagen fibrils interact with phosphatidylcholine and phosphatidylglycerol vesicles. Fluorescence polarization of (1,6-diphenyl-1,3,5-hexatriene) DPH-labeled vesicles, circular dichroism and differential scanning calorimetry studies have been performed. The protein-lipid interaction produces a decrease of the enthalpy of the phospholipid phase transition. Positive charges of lysine residues of the protein are involved in the interaction as experiments with succinylated collagen show. The kinetic parameters and the extent of the fibrillogenesis of collagen are modified by the phospholipid vesicles.

Key words: protein-lipid interaction, type I collagen.

Introduction

Although there are many evidences of the in vivo association between collagen fibers and lipids (Noble and Boucek, 1955; Bonner et al., 1975; Tall et al., 1978; Le Lous et al., 1982; Barnes, 1985), molecular studies at this level have rarely been done. We have previously described the interaction between monomeric type I collagen and phospholipid vesicles, from a molecular point of view (Martinez del Pozo et al., 1988). This study was performed at acid pH in order to minimize protein-protein interactions. Collagen behaves as a peripheral protein against lipid vesicles, according to such studies. However, at neutral pH and other physiological conditions when fibrillogenesis occurs, the picture may become sensibly different. In fact, the interaction occurs between supramolecular organizations-collagen fibrils and lipid bilayers. Thus, the molecular study of such interactions under physiological conditions would be very interesting in spite of the number of methodological problems derived from the low solubility of the system. This analysis is the aim of this paper.

Experimental procedures

Preparation of collagen samples

Type I collagen was isolated from rat tail tendons as previously described (Chandrakasan et al., 1979; Martinez del Pozo et al., 1988). The isolated protein was shown to be pure and consisted of largely monomeric type I collagen, according to its amino acid composition, SDS-polyacrylamide gel electrophoresis pattern, circular dichroism spectrum and chromatographic behavior under denaturing conditions (Chandrakasan et al., 1979; Olmo et al., 1985). The intactness of the non-helical ends of the protein was confirmed by determining its tyrosine content (Martinez del Pozo et al., 1986a, b). The purified protein was analyzed for its lipid content (Bligh and Dyer, 1959; Gavilanes et al., 1979). This content was negligible (0.08% w/w) for the present studies.

Succinylated collagen was prepared by adding solid succinic anhydride (1:1 w/w) protein to reagent) to a rapidly stirred solution of the protein in 0.15 M phosphate buffer, pH 7.5, at room temperature. The pH was maintained at
8.0 by addition of a NaOH solution. The reaction mixture was maintained at room temperature for 1 h. The protein derivative was isolated by dialysis and lyophilized. The number of modified residues was evaluated by amino acid analysis, after dinitrophenylation of the succinylated protein.

The collagen fibrils were formed in 30 mM Tris, 30 mM Na$_2$HPO$_4$, 0.2 M NaCl, 2.5 mM acetic acid, pH 7.0-7.2, by mixing a collagen stock solution in 5 mM acetic acid with the required volume of the corresponding salt solution. The final protein concentration was 0.1 mg/ml. The fibril formation was monitored in a Beckman DU-8 spectrophotometer by using micro-cells in a thermostatted cell holder ("Tm compuset" accessory, Beckman). Samples were loaded at room temperature and rapidly heated up to 30°C. The turbidity of the samples was continuously recorded by measuring absorbance at 315 nm. The resulting fibril formation curves were analyzed for both (A) the maximum absorbance variation and (t½) the half-time of the process. The above reaction mixture was maintained for 2 h in order to obtain collagen fibrils for further experiments.

**Preparation of lipid vesicles**

Synthetic phospholipids, dipalmitoyl- and dimyristoylphosphatidylcholine (DMPC and DPPC) and dimyristoylphosphatidylglycerol (DMPG), were purchased from Avanti Polar Lipids (Birmingham, AL) and their homo-geneity assayed by thin-layer chromatography (Gavilanes et al., 1981). The lipid vesicles were prepared in 30 mM Tris-HCl buffer, pH 7.0, containing 0.1 M NaCl, at 1 mg/ml phospholipid concentration, for 30 min in a water bath sonifier. The temperature during the process was maintained above the phase transition of the corresponding phospholipid. Lyosphospholipids were not observed after the preparation of the lipid vesicles.

**Spectroscopic measurements**

Absorbance determinations were performed on either Cary 118 or Beckman DU-8 spectrophotometer. The absorbance values were corrected for the light-scattering of the samples when required (Gavilanes et al., 1985).

Fluorescence measurements were carried out on a Perkin Elmer MPF 44E spectrofluorimeter operated in the ratio mode. Cells of 0.2-cm optical path were used. The labeling of the phospholipid vesicles was performed as previously described (Gavilanes et al., 1981; 1985). The polarization degree (P) of the fluorescence emission of 1,6-diphenyl-1,3,5-hexatriene (DPH) was determined as described (Martinez del Pozo et al., 1988). These measurements were corrected for the light-scattering of the samples according to Lentz et al. (1979). Microviscosity (η) was calculated from the (P) value according to Shinitzky and Barenholz (1978). The relative activation energy of the microviscosity was determined from the corresponding Arrhenius plot (Lentz et al., 1978).

Circular dichroism spectra were obtained on a Jobin Yvon Mark III dichrograph fitted with a 250-W xenon lamp. The spectra were recorded at 0.2 nm/sec scanning speed in 0.05 or 0.01 cm optical-path cells. The results were calculated in units of degree × cm$^2$ × dmol$^{-1}$, considering 91.2 as mean residue weight for collagen (Piez and Sherman, 1970).

**Other analytical procedures**

Differential scanning calorimetry (DSC) was performed as previously described (Martinez del Pozo et al., 1988). The protein concentration was determined by amino acid analysis (in constant boiling, 5.7 N HCl, containing 0.1% (w/v) phenol, in evacuated and sealed tubes) on a Durrum D-500. The phospholipid concentration was determined according to (Rousser et al., 1966).

**Results and Discussion**

We described that type I collagen from the tail tendon of young rats interacts with phospholipid vesicles. This study was performed at acid pH. Thus, the results were mainly related to monomeric collagen since protein aggregation is minimized for such conditions. The observed interaction only involved the triple helical part of the collagen molecule, the telopeptides being not involved because of identical results were obtained for native and pepsin plus carboxypeptidase Y -treated collagens. The phospholipid vesicles-collagen complex was proposed to be maintained by electrostatic interactions between the zwitterionic polar heads of phosphatidylcholine, the phospholipid used for such a study, and the amino acid side chains of the protein (Martinez del Pozo et al., 1988).

At neutral pH$_{5}$ collagen forms fibrils and thus creating new structural features involving the surface charge distribution of the molecule. The structure may result in modification of the reported electrostatic interaction with the lipid component.

Type I collagen also interacts with phosphatidylcholine vesicles at neutral pH$_{5}$ (Figure 1). DPH-labeled DMPC vesicles were used for this study. At lipid to protein molar ratios up to 250:1, preformed collagen fibrils do not significantly modify the transition temperature value of the phospholipid vesicles but slightly decreases the activation energy of the microviscosity (about 15-20% decrease; Fig. 1). This result is similar to that obtained at acid pH for monomeric collagen, although the activation energy was decreased 40% upon interaction at acid pH (Martinez del Pozo et al., 1988). Thus, the perturbation on the lipid
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Fig. 1. (A) Arrhenius' plot for the microviscosity ($\eta$) of DPH-labeled DMPC vesicles. Microviscosity values (average ± SD corresponding to three different experiments) were determined from the polarization degree as described in Experimental Procedures section. (---) Phospholipid alone; (---) collagen fibrils-DMPC vesicles (0.2 mg/ml of collagen; 250:1 lipid to protein molar ratio). The temperature is expressed in °K. (B) Variation of the activation energy of the microviscosity ($\Delta H$) versus temperature and expressed in arbitrary units. Maxima appear at the corresponding temperature phase transition. Samples were analyzed in 30 mM Tris-HCl buffer, pH 7.0, containing 0.1 M NaCl.

The bilayer produced by collagen fibrils is lower than that produced by monomeric collagen.

The presence of collagen fibrils also modifies the microviscosity of DPH-labeled DMPC-DPPC mixed vesicles. This population of vesicles exhibits a transition temperature between the two extreme values corresponding to each pure phospholipid (23 °C for DMPC and 41 °C for DPPC). The change produced on the phase transition of DMPC-DPPC is explained by a preferential interaction of the protein with the liquid-crystalline phase of the phospholipid mixture. This can be deduced from the results in Figure 2 where the effect of collagen on the phase transition of DMPC-DPPC vesicles is given.

DMPC vesicles do not affect the triple helical conformation of collagen, based on the absence of ellipticity variations by circular dichroism measurements at 220 nm (the characteristic positive band of type I collagen appears at this wavelength) (data not shown). This was also observed at acid pH (Martinez del Pozo et al., 1988).

The study of the effect of succinylated collagen on DMPC vesicles at neutral pH was also performed. In this case no interaction was detected. The profile of the phospholipid

Fig. 2. (A) Arrhenius’ plot for the microviscosity of DPH-labeled DMPC-DPPC vesicles (66:33% molar). (---) Phospholipid vesicles; (---) collagen fibril-phospholipid vesicles complex (0.2 mg/ml collagen; 250:1 total lipid to protein molar ratio). (B) Variation of the activation energy of the microviscosity versus temperature. Other experimental details are those indicated in the legend of Fig. 1.

Fig. 3. (A) Enthalpy changes of the phase transition of DMPC vesicles as a function of the total collagen present. Values are expressed as percentages (average ± SD corresponding to two different experiments) of the enthalpy obtained for the lipid alone. The phospholipid concentration was 0.5 mg/ml. The 100% of the enthalpy change was 5.7 kcal/mol. (B) Half-time ($t_{1/2}$, min) of the fibrillogenesis of collagen (see Experimental Procedures) versus protein concentration, this study being performed at 30 °C in 30 mM Tris-HCl, 30 mM Na$_2$HPO$_4$, 2.5 mM acetic acid, pH 7.0—7.2, containing 0.2 M NaCl.
phase transition, determined by fluorescence polarization measurements, is not changed (data not shown). This result demonstrates the involvement of the positive charges of the lysine residues of collagen in the protein-lipid interaction at neutral pH.

The analysis of the collagen-lipid interaction by using fluorescence polarization measurements cannot be performed at high proportions of collagen fibrils for the phospholipid vesicles concentration used. The turbidity of the system (due to the presence of fibrils, phospholipid vesicles and lipid-fibril complexes) is too high and the validity of the corrections for light-scattering becomes doubtful. This problem did not appear under acid conditions because of the monomeric character of collagen at such a pH value.

In order to avoid the spectroscopical troubles arising from the light-scattering of the samples, we performed a titration of the phospholipid vesicles population with a collagen solution by using differential scanning calorimetry. The obtained results are given in Figure 3A. The enthalpy of the phospholipid phase transition (from the gel-crystalline to liquid-crystalline phases) is decreased by the protein, but a saturating effect is not observed. The enthalpy decreases up to a lipid/protein molar ratio of about 2000:1 and increases for higher protein/lipid molar ratios (Fig. 3A). The titration plot obtained by DSC measurements at acid pH exhibited an hyperbolic character with saturation around 900: 1 lipid to protein molar ratio (Martinez del Pozo et al., 1988). A similar saturating behavior would be expected at neutral pH. However, the obtained titration plot strongly suggests the existence of two different processes. One would be equivalent to the modification occurring at acid pH which would be reversed by a second process predominant at higher collagen concentrations.

In order to explain the results in Fig. 3A, we studied the fibrillogenesis of collagen under identical conditions to those used on the DSC measurements. The result is given in Figure 3B. From the comparison of the two curves in Fig. 3, the results thus obtained by the DSC analysis can be interpreted in terms of competition between the two following processes: phospholipid vesicles-collagen interaction and collagen-collagen interaction to form fibrils, being the last one the predominant at high collagen concentration. This competition was not observed at acid pH because collagen aggregation is minimized under such conditions.

Fibrils also interact with DMPC since an enthalpy change for the phase transition of the phospholipid is observed even at high collagen concentration (about 15% of the maximum variation observed; Fig. 3A).

To verify the preponderance of collagen aggregation against DMPC-collagen interaction, we studied the effect of phospholipids on the fibrillogenesis of collagen by measuring the absorbance variation at 315 nm (Figure 4). If fibril formation is the predominant process as the results of Fig. 3 suggest, the influence of the DMPC vesicles on the collagen fibrillogenesis would not be very significant. In fact, DMPC vesicles up to about 4000: 1 lipid protein molar ratio do not significantly affect the characteristic parameters of the collagen fibrillogenesis. The final absorbance variation (Fig. 4B), which would be related to the fibril size, is not modified upon the presence of DMPC vesicles. The t½ is only about 15% decreased on the presence of lipid vesicles (the enthalpy change of the lipid phase transition at high collagen concentration is about 15% of the maximum variation). These results are in agreement with the predominance of collagen fibrillogenesis against collagen-DMPC vesicles interaction.

Based on the observed involvement of the positive charge of lysine residues of collagen on the interaction with the lipid vesicles, we studied DMPC vesicles. This is an acid phospholipid at neutral pH, which exhibits a different behavior than the zwitterionic phospholipid DMPC. Type I collagen fibrils also interact with DMPC vesicles (Figure 5). The extent of the interaction is higher than that observed for DMPC vesicles. The cooperative character of the phospholipid phase transition is significantly decreased by the fibrils (Fig. 5).

Moreover, these acid phospholipid vesicles interfere with the collagen fibrillogenesis (Fig. 4). The t½ value of the process is increased from 70 to about 110 min at 400: 1 lipid to protein molar ratio under our experimental conditions. The absorbance variation at 315 nm is also decreased. Thus, the rate of fibrillogenesis as well as the size of the collagen fibrils are diminished by the lipid vesicles.
Different behavior for DMPC and DMPG can be concluded from these experiments. Type I collagen fibrils interact with both types of phospholipid vesicles. If we consider that the competition between protein-lipid interaction and collagen fibrillogenesis is an index of the affinity of the protein for the vesicles, the type I collagen fibrils would exhibit a higher affinity for DMPG than for DMPC at neutral pH, which would be related to the acid character of DMPG against the zwitterionic character of DMPC.

Collagen fibril formation is inhibited by ionic and non-ionic surfactants (Suarez et al., 1980) which would be interpreted in terms of weakening of hydrophobic interactions between collagen molecules. The inhibition of fibril formation by an excess of phosphate anion has been reported (Williams et al., 1978). This has been explained by the phosphate moiety of the phospholipid because of the different results observed for DMPC and DMPG vesicles, but it has an electrostatic component which modifies the profile of the phase transition of the vesicles, thus exhibiting its own character. In summary, the interaction of collagen with lipid produces alteration on the fibrillogenesis of the protein. Thus, collagen-phospholipid interaction may have physiological consequences at level of the extracellular matrix organization.

References


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