Molecular Crystals and Liquid Crystals

Publication details, including instructions for authors and subscription information:
http://www.tandfonline.com/loi/gmcl20

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Version of record first published: 18 Mar 2009

To cite this article: Michele Francesco Maria Sciacca, Vincenzo Carbone, Matteo Pappalardo, Danilo Milardi, Carmelo La Rosa & Domenico Maria Grasso (2009): Interaction of Human Amylin with Phosphatidylcholine and Phosphatidylserine Membranes, Molecular Crystals and Liquid Crystals, 500:1, 73-81

To link to this article: http://dx.doi.org/10.1080/15421400802713710

Mol. Cryst. Liq. Cryst., Vol. 500, pp. 73–81, 2009

ISSN: 1542-1406 print/1563-5287 online
DOI: 10.1080/15421400802713710

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Interaction of Human Amylin with Phosphatidylcholine and Phosphatidylserine Membranes

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We studied the interaction of human amylin with dipalmitoylphosphatidylcholine and dipalmitoylphosphatidylserine vesicles using differential scanning calorimetry, circular dichroism and molecular modelling. The results suggest that inappropriate insertion of amylin into the hydrophobic core of a lipid bilayer could hinder the secretion of amylin from pancreatic β-cells.

Keywords: amylin; circular dicroism; differential scanning calorimetry; lipid bilayer; molecular modeling

INTRODUCTION

Diabetes mellitus type II, or non-insulin-dependent diabetes mellitus, is a protein conformational disease characterized by dysfunction and progressive damage of Langherans' β cells in the pancreas [1–4]. The disease is accompanied by deposition of amyloid fibrils mainly composed of the islet amyloid polypeptide precursor, or amylin, and a decrease in the blood level of this peptide hormone [5–7]. Amylin, co-stored and co-secreted with insulin by Langerhans' β cells, is a 37 amino acid residue polypeptide that normally plays an important role in triggering glycogen metabolism [8–9]. One potential mechanism of

This research was financially supported by MIUR (FIRB Ñ RBNE03PX83, PRIN 2005035119002) and University of Catania.

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amylin pathogenicity could be an abnormal interaction of amylin with lipid membranes [10–12] that is accompanied by an increase in the rate of fibrillogenesis dependent on membrane charge, pH, and amylin concentration [13]. In physiological conditions amylin is positively charged at two positions, lysine-1 and arginine-11, has a partial positive charge on histidine-17 and an irregular distribution of hydrophobicity/hydrophilicity. From an electrostatic point of view, amylin can be considered a macrodipole. It is possible that the balance between hydrophobic and electrostatic interactions drives the protein/lipid interaction of amylin with the cellular membrane. These interactions would depend on the nature of the phospholipids and on the electrostatic potential of the surface. During trafficking in the cytosol, amylin is transported by phospholipid vesicles made of zwitterionic (dipalmitoilphosphatidylcholine [DPPC] and sphingomyelin phoshatidylethanolamine [DPPE]) and negatively charged lipids (dipalmitoylphosphatidylserine [DPPS] and phosphatidylinositol [PPI]). Abnormal lipid/membrane interactions could disrupt normal amylin trafficking and negatively affect the availability of amylin to the extra cellular compartments, thereby nucleating amyloid disease prior to β-cell death. Here we investigate the interaction of amylin with large unilamellar vesicles (LUVs) composed of DPPC or DPPS using differential scanning calorimetry (DSC), circular dichroism (CD), and molecular modelling (MM), in order to determine the effects of amylin on the lipid membrane properties and in turn, the membrane-induced conformational transitions of this hormone.

EXPERIMENTALS

Materials

DPPC and DPPS were purchased from Genzyme Corporation (Cambridge, MA, USA). Human amylin (purity > 99%) was purchased from Bachem (Bubendorf, Switzerland), and used without further purification. $Ca(NO_3)_2 \cdot 4H_2O$ and other inorganic salts (99.9% purity) used to prepare phosphate buffer solutions were obtained from Sigma Chemical Co. (St. Louis, MO, USA).

Methods

Vesicle Preparation

Model membranes were prepared as described elsewhere [14]. Briefly, solutions of pure phospholipids, in $CHCl_3$ or $CHCl_3/CH_3OH$ 3:1 mixture were dried under nitrogen and evaporated to dryness in

vacuum in round-bottomed flasks. The resulting lipid film was hydrated in an appropriate volume of buffer and dispersed by vigorous stirring in a water bath set at 4°C above the gel-liquid crystal transition temperature of the membrane. The final nominal concentration of the lipid was 0.2 mM. In order to obtain LUVs, the multilamellar vesicles so obtained were extruded through polycarbonate filters (pore size ¼ 100 nm; Nuclepore, Pleasanton, CA,) mounted in a mini-extruder (Avestin, Inc., Ottawa, Canada) fitted with two 0.5 ml Hamilton gastight syringes (Reno, NV, USA). Usually we subjected samples to 23 passes through two filters in tandem as recommended elsewhere [15]. An odd number of passages were performed in order to avoid contamination of the sample by vesicles that might not have passed through the filter. Using this protocol, we prepared LUVs of pure DPPC and LUVs of pure DPPS.

Differential Scanning Calorimetry

DSC runs were performed using a VP-DSC instrument (MicroCal, Northampton, MA). We performed all experiments at a scan rate of $1^{\circ}C\,min^{-1}$ in a 0.5 ml cell. An extra external pressure of about 2 bars was applied to the solution. In order to obtain the excess heat capacity curves (*C*pexc), the DSC curves were subtracted from a baseline obtained by a third order polynomial fit of the pre- and post-transition *C*p trends after instrument baseline correction. Samples were prepared by mixing unilamellar liposomes with the peptide solution. Samples were shaken vigorously before they were loaded into the calorimeter. The final lipid concentration was 0.2 mM and the lipid: peptide molar ratio was 20:1. The liposome suspensions were scanned before and after addition of the peptide and the samples were equilibrated at the starting temperature for 30 minutes.

Circular Dichroism

CD measurements in the far-UV region (200–330 nm) were performed with a JASCO 700 spectropolarimeter using quartz cuvettes of 0.1 cm optical path. To prevent scattering, all measurements were performed at a temperature above gel/liquid crystal transition. All the other experimental conditions were the same used for DSC experiments. Fifteen accumulations were performed for every CD spectra, Final CD spectra were obtained by subtracting the baseline spectra of the pure lipid.

Molecular Modelling

In order to perform calculations, we made the following conformational assumptions for amylin: an alpha helix conformation is adopted

when the hormone interacts with DPPS surface, except for residues 1 through 8 which are involved in a disulfide bridge [13] and, according to Steven's model [16], amylin adopts a partial β-sheet conformation when it interacts with DPPC membranes. Protons were added in order to account for the ionization equilibrium of the residues at pH 7.

The Forcefield Charmm22 was used for all the calculations. Adaptive Poisson-Boltzmann Solver software was used to calculate electrostatic potential around the protein by resolving the Poisson- Boltzmann equation [17] in three dimensions and adopting a dielectric constant value of 2 for protein [18], 80 for water [19], and 2 for the hydrophobic core of membrane [20]. All calculations were carried out using full Coulombic approximation. The grid used for electrostatic potential calculations had a density of 169 points per $\mathring{A}^2$. Ionic strength was maintained constant at a value of 10 mM. Hydrophobicity of amylin was estimated using the Kyte-Doolittle method [21].

RESULTS AND DISCUSSION

We analyzed the heat capacity and phase transition temperature changes at the gel-liquid crystal thermal transition of lipid/peptide systems and the corresponding CD spectra to clarify the nature of the lipid/peptide interactions and the topological arrangement of the peptide when it interacts with the lipid matrix. The enthalpy change observed during the lipid gel-liquid crystal transition is strongly influenced by the extent of the interaction between guest molecules and the hydrophobic tails of lipids [20,22]; conversely, melting temperature (T_m) changes are related to electrostatic lipid/guest interactions at the interface [23]. Bearing this in mind, we performed DSC on LUVs of pure zwitterionic DPPC or negatively charged DPPS, and on LUVs in presence of amylin protein. The use of a nanocalorimeter allowed us to use the same very dilute solution adopted for the spectroscopic measurements, minimizing the risk of protein aggregation. Panel A of Figure 1, illustrates the DSC curves related the thermal transitions of LUVs of pure DPPC and DPPC/amylin systems. The corresponding CD spectra are shown in Panel B.

Panel A of Figure 2, shows the DSC curves obtained by heating LUVs of pure DPPS and DPPS/amylin systems. The corresponding CD spectra are shown in Panel B. The whole of the calorimetric and CD results are presented in Table 1. The calorimetric data demonstrates that, with respect to pure DPPC, the presence of amylin induces a decrease in the ΔH of gel-liquid crystal transition of about $9\,kJ\,mol^{-1}$ without any significan change in the T_m. If one considers that for each mole of blocked CH_2 rotamers the transition energy

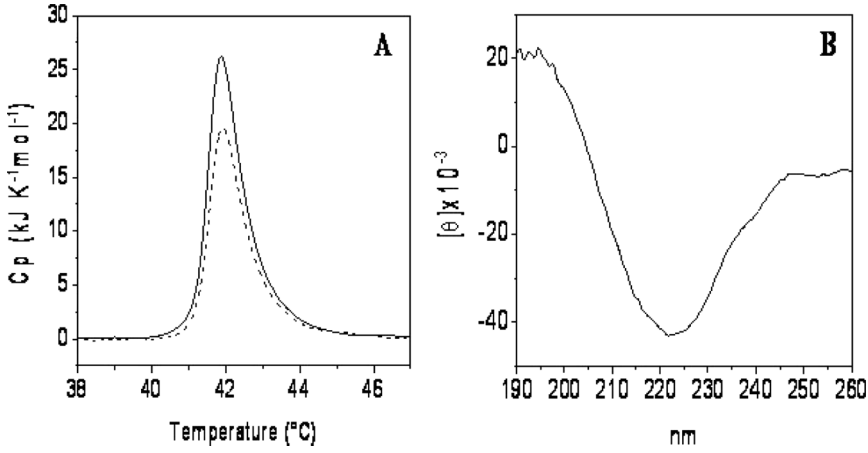

FIGURE 1 Panel A shows thermograms of LUV DPPC (solid line), LUV DPPC/hIAPP 20:1 (dashed line). Panel B CD profile of DPPC/hIAPP 20:1.

decreases of about 1 kJ [20], DSC data suggest that the interaction between amylin and the DPPC bilayer occurs mainly in the interior of hydrophobic chains blocking, on average, the free rotation of 9/36 rotamers. Moreover, the absence of variation in T_m shows that the interaction between the protein and the zwitterionic lipids does not significantly involve the surface of the bilayer. The spontaneous

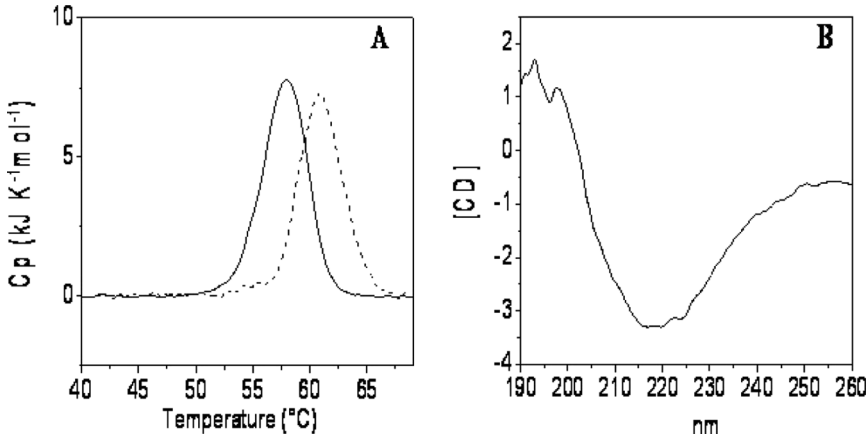

FIGURE 2 Panel A shows thermograms of pure DPPC (solid line), DPPS/hIAPP 20:1 (dashed line). Panel B shows CD of DPPS/hIAPP 20:1.

TABLE 1 Calorimetric Data Referring to DPPC and DPPS Systems

System	T_m (°C)	ΔH (kJ mol^{-1})	$\Delta\Delta H$ (kJ mol^{-1})*	Conformation
DPPC	41.8 ± 0.1	37.9 ± 1.9	–	–
DPPC + hIAPP 20:1	41.9 ± 0.1	29.0 ± 1.4	−8.9	β-sheet
DPPS	57.9 ± 0.1	38.6 ± 1.9	–	–
DPPS + hIAPP 20:1	60.9 ± 0.1	36.1 ± 1.8	−2.5	α-helix→β-sheet[a]

[a]Initially hIAPP adopts an α-helix conformation, but rapidly converts into β-sheet structure [13].

insertion of amylin into the hydrophobic core of the DPPC is supported by the electrostatic distribution and hydrophobic characteristics of amylin in a partially β-sheet conformation (Fig. 3 Panel A). CD spectra, reported in Panel B of Figure 1, suggest the presence of β-sheet rich structure, as evidenced by a minimum at 220 nm. Comparing CD data with DSC results, it is possible to hypothesize a mechanism in which hIAPP interacts with hydrophobic core of DPPC LUV adopting a structure which is compatible with the formation of amyloid fibrils. The ΔH corresponding to gel-liquid crystal phase transition of the amylin/DPPS system is only 2.5 kJ mol^{-1} less than pure DPPS, while the Tm changes markedly, passing from 57.9 to 60.9°C. These data can be explained by assuming a very weak interaction of

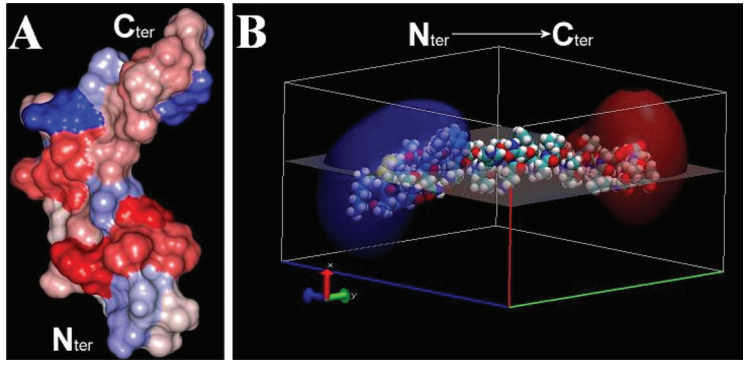

FIGURE 3 Panel A shows the hydrophobicity of hIAPP in a β-sheet conformation calculated by kyte-doolittle method. Red indicates hydrophobic residues, blu indicates hydrophilic residues. Panel B shows electrostatic field of hIAPP in an α-helix conformation, calculated by Poisson-Boltzmann equation. Red indicates negative isopotential surface,while blue indicates positive isopotential surface.

polypeptides with the hydrophobic region of the bilayer and an electrostatic interaction between the negatively charged surface of the lipid and the positive charges of the polypeptide (Fig. 3 Panel B). Figure 2 panel B shows the CD spectra of the DPPS/hIAPP 20:1 sample. The minimum at 220 nm is indicative of a β-sheet conformation. However literature reports that negatively charged lipid promotes an initial α-helical conformation that spontaneously converts into a β-sheet structure [13]. All these data report about a different behaviour for DPPC/amylin versus DPPS/amylin systems. In the DPPC/amylin system, where the lipid surface is zwitterionic, the peptide-membrane interaction is driven by hydrophobicity and the protein crosses the hydrophilic interface of lipid membrane to interact with the hydrophobic core of lipid, where it assumes a β-sheet conformation. In the DPPS/amylin system where the lipid surface is negatively charged, the peptide-membrane interaction is driven by the electrostatic forces; the protein does not penetrate the electrostatic barrier and interacts only with the surface of the membrane. In order to assess if membrane induces aggregation and catalyzes amyloid fibrils formation we performed some optical microscopy experiments. Samples were treated with Congo Red and visualized in polarized light with the aim to evidence the typical green birefringence of amyloid fibrils. Figure 4 is an image obtained at 500X magnification by an optical Zeiss polarizing

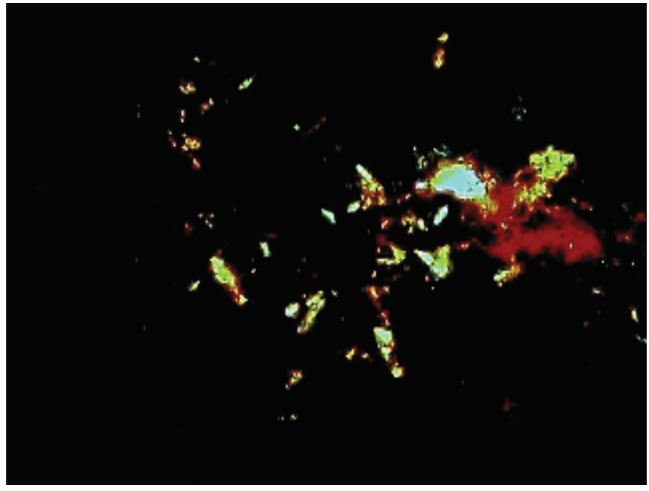

FIGURE 4 Image of LUV DPPC/hIAPP 20:1 system stained with congo red in polarized light, obtained with optical Zeiss polarizing microscope (magnification 500X). This image shows tipical birefringence of amyloid structure.

microscope that shows the presence of fibrils in the DPPC/amylin system, the green stain indicates area rich of amyloid deposits, other zones are black due to a filtering system. On the other hand, no fibrils are evident in the DPPS/amylin system.

CONCLUSIONS

Our results show that amylin inserts spontaneously into the hydrophobic core of DPPC lipid bilayer where it assumes a β-sheet conformation. Contrarily, the interaction of amylin with the negatively charged DPPS membranes is driven by electrostatic forces, and the protein interacts with the lipid surface where it initially assumes an α-helix conformation and then rapidly converts into a β-sheet structure [13]. These evidences support the hypothesis that the insertion of amylin into the hydrophobic core of the carrier vesicles could result in the entrapment of amylin into the cell membrane during exocytosis, thereby resulting in a deficiency in the level of circulating amylin and in the formation of β-sheet structures that are the primary step in protofibril formation. In particular, under our experimental conditions, amylin spontaneously inserts into the highly hydrophobic and anisotropic core of DPPC, where it accumulates, and ultimately forms β-sheet aggregates. In presence of negatively charged vesicles (DPPS) the lipid-peptide interaction involves only the surface of the LUV.

Our evidence suggests that "*in vivo*," where carrier vesicles are composed of both zwitterionic and charged lipids, any factor that perturbs the electrostatic environment could lead to an abnormal insertion and consequent storage of amylin in the hydrophobic core of carrier vesicles. Further, our results suggest that: a) molecular research regarding the protein aggregation in misfolding diseases should be directed towards lipid-protein interactions rather than focusing on the protein alone, and b) particular attention should be focused on the study of factors that influence the "electrostaticity" of the lipid/protein system, such as pH, ionic strength, and the presence of specific ions.

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