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ScienceDirect
Biochimica et Biophysica Acta 1778 (2008) 198–205
Biochimica et Biophysica Acta BBA
www.elsevier.com/locate/bbamem

Cholesterol and anionic phospholipids increase the binding of amyloidogenic transthyretin to lipid membranes

Xu Hou [a], Adam Mechler [b], Lisandra L. Martin [b], Marie-Isabel Aguilar [a], David H. Small [a,c,*]

[a] Department of Biochemistry and Molecular Biology, Monash University, Clayton, VIC 3800, Australia
[b] School of Chemistry, Monash University, Clayton, VIC 3800, Australia
[c] Menzies Research Institute, Hobart, Tasmania 7000, Australia

Received 5 July 2007; received in revised form 5 September 2007; accepted 21 September 2007
Available online 29 September 2007

Abstract

Deposition of transthyretin (TTR) amyloid is a pathological hallmark of familial amyloidotic polyneuropathy (FAP). Recently we showed that TTR binds to membrane lipids via electrostatic interactions and that membrane binding is correlated with the cytotoxicity induced by amyloidogenic TTR. In the present study, we examined the role of lipid composition in membrane binding of TTR by a surface plasmon resonance (SPR) approach. TTR bound to lipid bilayers through both high- and low-affinity interactions. Increasing the mole fraction of cholesterol in the bilayer led to an increase in the amount of high-affinity binding of an amyloidogenic mutant (L55P) TTR. In addition, a greater amount of L55P TTR bound with high affinity to membranes made from anionic phospholipids, phosphatidylglycerol (PG) and phosphatidylserine (PS), than to membranes made from zwitterionic phospholipid phosphatidylcholine (PC). The anionic phospholipids (PS and PG) promoted the aggregation of L55P TTR by accelerating the nucleation phase of aggregation, whereas the zwitterionic phospholipid PC had little effect. These results suggest that cholesterol and anionic phospholipids may be important for TTR aggregation and TTR-induced cytotoxicity.

Keywords: Transthyretin (TTR); Cholesterol; Phospholipid; Aggregation; Amyloid; Binding

1. Introduction

Neurodegenerative diseases associated with amyloidosis involve the build-up of proteinaceous deposits in the extracellular compartment of the nervous system [1,2]. Although the amyloidogenic proteins in their native state have little similarity in structure and perform different biological functions, the common structure of amyloid fibrils has led to the suggestion that the mechanisms of protein aggregation and neurotoxicity may be similar for different amyloidoses [3–5].

Familial amyloidotic polyneuropathy (FAP) is a fatal hereditary neuropathy characterized by systemic deposition of amyloid formed from misfolded transthyretin (TTR) [6,7], a transporter protein for thyroxine (T_4) and retinol in the plasma and the brain [8]. Native TTR consists of four identical subunits, each containing 127 amino-acid residues which form a predominantly β-sheet structure [9]. A large number of mutations have been identified in TTR, most of which cause amyloidosis [7,10]. It has been suggested that amyloidogenic mutations destabilize the tetrameric structure of TTR, leading to the formation of an amyloidogenic intermediate (probably a TTR monomer) which can self-assemble to form amyloid [11,12]. Although the mechanisms of TTR-induced cytotoxicity are still poorly understood, previous studies have reported that amyloidogenic TTR may cause its cytotoxic effects by disrupting intracellular Ca^{2+} homeostasis [13], inducing

Abbreviations: Aβ, β-amyloid protein; AFM, atomic force microscopy; DLS, dynamic light scattering; ER, endoplasmic reticulum; FAP, familial amyloidotic polyneuropathy; HOPG, highly oriented pyrolytic graphite; K_D, dissociation constant; PC, dimyristoyl-L-α-phosphatidylcholine; PE, dimyristoyl-L-α-phosphatidylethanolamine; PG, dimyristoyl-L-α-phosphatidylglycerol; PrP, prion protein; PS, dimyristoyl-L-α-phosphatidylserine; RU, response unit; SM, sphingomyelin; SPR, surface plasmon resonance; SUVs, small unilamellar vesicles; T_4, thyroxine; TTR, transthyretin; VGCCs, voltage-gated Ca^{2+}-channels; WT, wild-type

* Corresponding author. Menzies Research Institute, Hobart, Tasmania 7000, Australia. Tel.: +61 3 9905 1563; fax: +61 3 9905 3726.
E-mail address: David.Small@med.monash.edu.au (D.H. Small).

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doi:10.1016/j.bbamem.2007.09.018

endoplasmic reticulum (ER) stress [14] and up-regulating pro-inflammatory cytokines [15]. Several studies have identified TTR oligomers as the major form of TTR that cause cytotoxicity [13,16].

Recently, we reported that TTR binds predominantly to lipids in the plasma membrane via electrostatic interactions, and that this binding is correlated with the cytotoxic effect of amyloidogenic TTR [17]. Previous studies of other amyloidogenic proteins have also shown that binding to lipids in the plasma membrane is an important step in the aggregation and cytotoxicity [18,19]. It has been suggested that binding to membrane lipids may induce protein misfolding and aggregation by providing a favorable local environment [20–22]. Variations in lipid composition of the membrane can modify the protein–lipid interactions. For example, the concentration of cholesterol is correlated with the membrane binding of amyloidogenic proteins, such as β-amyloid protein (Aβ) [19,23] and prion protein (PrP) [18]. Cholesterol has also been identified as a major component of the lipids that are associated with various amyloid deposits [24]. It has been suggested that membrane-bound Aβ [25] or PrP [26] may act as a nucleus for aggregation. By modulating the organization of the plasma membrane, cholesterol may contribute to the aggregation [27–29] and cytotoxicity [18,19,27] of amyloidogenic proteins. In addition, anionic phospholipids have also been reported to be involved in the binding of some amyloidogenic proteins [22,30,31]. It is not known which lipid species in the membrane preferentially bind TTR, and how lipid composition influences this binding.

In the present study, the effects of cholesterol and different phospholipids on the binding and aggregation of TTR were studied. We show that the binding of an amyloidogenic TTR mutant, L55P TTR, to phospholipid bilayers is influenced by both the concentration of cholesterol and type of phospholipids in the membrane. We also show that anionic phospholipids promote the aggregation of L55P TTR. These results suggest that alterations in membrane lipid composition may play a role in the aggregation and cytotoxicity of TTR.

2. Materials and methods

2.1. Materials

The wild-type (WT) TTR and a highly amyloidogenic mutant, L55P TTR, were expressed in *Escherichia coli* and the native tetramers purified using ion-exchange and size-exclusion chromatography to a purity of >95% [17]. Dimyristoyl-L-α-phosphatidylcholine (PC), dimyristoyl-L-α-phosphatidylserine (PS), dimyristoyl-L-α-phosphatidylglycerol (PG), and dimyristoyl-L-α-phosphatidylethanolamine (PE) were purchased from Avanti Polar Lipids (Alabaster, AL). Highly oriented pyrolytic graphite (HOPG) of ZYB quality was the product of Advanced Ceramics (Cleveland, OH). Sphingomyelin (SM) and cholesterol were purchased from Sigma-Aldrich (Castle Hill, NSW, Australia).

2.2. Preparation of liposomes from phospholipids

Artificial liposomes with different compositions were prepared as described in a previous study [17]. Briefly, the lipids were dissolved in chloroform/methanol (3:1, v/v) at a concentration of 2 mM. Lipid mixtures were prepared with a composition based on that of the grey matter of human brain [32], except that the cholesterol concentration was 0, 25 or 50% on a molar basis of the total lipids, while the remaining lipids were in a constant molar ratio of 6:5:2:2 (PC:PE:PS:SM). The lipids were dried under a constant flow of N_2 gas, and then *in vacuo* overnight, to create a uniform lipid film which can form liposomes by hydration. The dried lipids were resuspended in 20 μM phosphate buffer (pH 7.4) containing 150 mM NaCl to a final concentration of 1 mM. After sonication for 30–60 min, the lipid suspension was extruded seventeen times through a polycarbonate filter membrane (50 nm pore size) using a LiposoFast extruder (Avestin, Ottawa, Canada) to produce unilamellar liposomes which were 50±4 nm in diameter as determined by dynamic light scattering (DLS) using a Zetasizer Nano ZS (Malvern Instruments, Malvern, UK). These small unilamellar vesicles (SUVs) were later used to prepare a lipid bilayer on the surface of biosensor chips for membrane binding studies [17,33].

2.3. Surface plasmon resonance analysis of membrane binding

The binding of TTR to lipid bilayers was studied in real time by surface plasmon resonance (SPR) using a Biacore 3000 biosensor (Biacore AB, Uppsala, Sweden) and was performed essentially as described previously [17]. Artificial liposomes were immobilized onto the surface of an L1 biosensor chip to form a lipid bilayer. TTR preparations (100 μl, in 20 mM phosphate buffer containing 150 mM NaCl, pH at 7.4) were centrifuged at 15,000×*g* for 1 min to remove insoluble materials, and then applied to the lipid bilayer at a flow rate of 30 μl/min for 200 s. The running buffer used was 20 mM phosphate buffer (pH 7.4) containing 150 mM NaCl. A 10-min wash by the running buffer was performed after each injection to allow for dissociation of TTR. The lipid bilayer was then regenerated by an injection of NaOH (10 μL of a 10-mM solution) at 50 μl/min. The amount of binding was determined by changes in the instrumental response unit (RU). As described previously [17], the amount of binding at equilibrium (R_{eq}), which represents the maximum binding of TTR at a given concentration, was calculated. The binding data were then analyzed using Scatchard plots in which R_{eq} was plotted against R_{eq}/C (where C=TTR concentration). Although TTR preparations at different aging times contained various forms of the protein with disparate molecular masses, TTR concentrations in all preparations were calculated by assuming that all TTR molecules were present as tetramers.

2.4. Analysis of TTR aggregation by solution turbidity assay and dynamic light scattering

TTR aggregation was monitored by changes in solution turbidity and particle size as TTR was incubated (aged) at 37 °C in various solutions. As described previously [13], solution turbidity was monitored by measuring the absorbance of the solution at a wavelength of 330 nm using a SmartSpec 3000 spectrophotometer (Bio-Rad, Hercules, CA). Changes in particle size were measured by means of DLS using a Zetasizer Nano ZS [13].

2.5. Atomic force microscopy

Atomic force microscopy (AFM) was used to examine changes in TTR morphology during the time course of aggregation and was performed as described in a previous study [13]. TTR solutions (20 μl, 0.1–1 μM) were deposited on a freshly cleaved HOPG surface and incubated at 37 °C for 30 min. After five washes with deionized distilled water, the HOPG surface was then dried under a constant flow of N_2 gas. TTR samples were examined by a Digital Instruments Nanoscope IV Multimode scanning probe microscope (Veeco Instruments, Woodbury, NY). NSC15 125 μm silicon cantilevers with a tip length of 15 μm (Mikromasch, Tallinn, Estonia) were used for data acquisition. Images were obtained in the tapping mode at oscillation frequencies of 200–300 kHz with constant adjustment of the force exerted on the cantilever. AFM images were analyzed by the WSxM 4.0 software (Nanotec Electronica S.L., Madrid, Spain).

3. Results

3.1. Effect of cholesterol on membrane binding of TTR

As previous studies have shown that cholesterol can increase the binding of Aβ to lipid membranes [19,28], we first examined