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Synergistic action of polyanionic and non-polar cofactors in fibrillation of human islet amyloid polypeptide

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Abstract **Recent studies have led us to suppose that synergistic action of multiple solute cofactors could play substantial roles in amyloid-type fibrillogenesis of pathogenic polypeptides. To support this view, we performed aggregation experiments of human islet amyloid polypeptide (IAPP) in media containing both polyanions and non-polar solvents. The results demonstrated that the fibrillation at sub-micromolar IAPP occurred only when polyanionic and non-polar solutes coexist. A simple sum of two independent cofactor's effects could not account for the synergistic action. We propose that this synergy of polyanionic and non-polar milieus could substantially modify the amyloidgenesis in the human body.**

Keywords: IAPP; Amyloid; Fibrillation; Alcohol; Polyanion; Synergistic effect

1. Introduction

Progressive formation of proteinaceous inclusions in brains and peripherals is a hallmark lesion of human degenerative diseases [1,2]. The amyloid-type aggregation of fibrillogenic polypeptides including β-peptide of Alzheimer's disease and islet amyloid polypeptide (IAPP) of type 2 diabetes has been related to pathological events responsible for cell death and organ dysfunctions [1–3]. Previous in vitro studies have elucidated molecular details of the fibrillation mechanism and the fibril structures [4–6], but the events occurring in the living body are largely unresolved.

Physiological concentrations of amyloidgenic proteins are lower than the critical lower limit concentrations of the fibrillation in vitro. To explain this discrepancy, previous studies have identified a variety of fibrillation enhancers such as lipid membranes [7,8], anionic surfactants [9,10] and polyanions [11–13]. Many species of solutes in physiological media, some being ionic and others being non-polar, could always coexist at any sites of the living body. This consideration had led us to suppose that synergy of different solute species might play substantial roles in the amyloidgenesis. In fact, the fibrillar deposits in apoptotic tissues often comprise glycosaminoglycans (GAGs), lipids, lipo-proteins and other organic molecules [3,4,13,14].

To support our proposition above, experiments were conducted in vitro for demonstrating a synergistic action of polyanionic and non-polar cofactors upon amyloid fibril formation of IAPP. Deposition of IAPP amyloid in human pancreatic islet is a common pathological feature of type 2 diabetes [3,14]. Each of polyanion and non-polar media is known to promote the IAPP fibrillation [14,15]. Heparin and alcohol were used for polyanion and non-polar cofactors, respectively. Heparin is a member of GAGs, abundant in the extracellular space of the living body, whereas alcohol is the most popular membrane mimicking solvent and is also known for enhancers of amyloidgenesis [16]. The aggregation process of IAPP was monitored by thioflavin T (ThT) binding, light scattering and transmission electron microscopy (TEM). The concentration of IAPP used here is lower than that of previous studies.

2. Materials and methods

Human IAPP was purchased from Peptide Institute Inc. (Osaka, Japan), and was disaggregated by a method of O'Nuallain and Wetzel [17]. Peptide solutions contained 20 mM Tris–HCl (pH 7.5), 0.1 M NaCl, 5 μM ThT, polyanion and alcohol. ThT, heparin, *N*-acetylheparin, poly-L-glutamate (polyE), 1,1,1,3,3,3-hexafluoroisopropanol (HFIP) and 2,2,2-trifluoroethanol (TFE) were purchased from Sigma Co. Other chemicals of reagent grade were purchased from Nacalai Tesque Co. (Kyoto, Japan).

Aggregation process was monitored by light scattering and ThT fluorescence. The sample solution was prepared in a quartz fluorescence cell (3 × 3 mm), sealed with parafilm, and kept at 8 °C. The cell was then transferred to a cell folder of a fluorescence spectrometer F-2500 (Hitachi, Japan). The cell folder was kept at 37 °C by a circulating water bath. Excitation/emission wavelengths and bandwidths were 340/340 nm and 2/2 nm for light scattering, and 440/480 nm and 5/10 nm for ThT fluorescence. The kinetic trace of the IAPP aggregation was fitted by a pseudo-first order function [18]:

$$F = F_0 + \frac{\Delta F}{1 + \exp[-(t - \varphi)/\tau]} \quad (1)$$

where F_0 and F are fluorescence intensities at time zero and t. Fitting parameters are ΔF, φ and τ. An initial delay time (t_d) and an aggregation rate in the evolution phase (k_{agg}) were determined by $k_{agg} = 1/\tau$ and $t_d = \varphi - 2 \cdot \tau$.

Morphology of aggregates was observed using an H-7000 electron microscope (Hitachi, Japan), operated at 75 kV. The sample solution was applied onto a carbon grid and stained with 2% uranyl acetate. Images were recorded on FG electron-microscopic films (Fuji film, Japan), developed in D-19 (Kodak, USA) for 3 min.

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Abbreviations: IAPP, human islet amyloid polypeptide; ThT, thioflavin T; HFIP, 1,1,1,3,3,3-hexafluoroisopropanol; TFE, 2,2,2-trifluoroethano; iPrOH, isopronanol; EtOH, ethanol; polyE, poly-L-glutamate; TEM, transmission electron microscopy; GAG, glycosaminoglycan

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3. Results and discussion

3.1. IAPP fibrillation in heparin and TFE solutions

Amyloid formation of IAPP was monitored by ThT fluorescence in solutions containing heparin and alcohol. The concentration of IAPP was 0.5 μM throughout. At this low peptide concentration, no aggregate was generated without cofactors even after incubation for 2 weeks. Typical aggregation kinetic curves were obtained at 4–8% TFE and 0.5–5 μg/ml heparin (Fig. 1A), which exhibited an initial delay followed by evolution and final plateau phases. Comparison of the kinetic profile of ThT fluorescence with that of normal-angle light scattering showed that the fluorescence intensity was a faithful measure of the amount of IAPP aggregates (Fig. 1B). Aggregates generated in these conditions were fibrous in shape (Fig. 1C), typical for amyloid.

Typical amyloid formation exhibits a nucleation-dependent kinetic property [19]. The initial aggregation delay observed in our case is a good indication of this kinetic type (Fig. 1A). To confirm it further, a seeding experiment was performed (Fig. 1D). Addition of the amyloid seeds to a freshly prepared sample significantly shortened the initial delay time, supporting that the aggregation of IAPP in the heparin–alcohol mixture proceeded in a nucleation-dependent fashion.

3.2. Synergistic action of heparin and TFE

Three kinetic parameters were determined from the aggregation kinetic profile. Those are an initial delay time (t_d), an aggregation rate in the evolution phase (k_{agg}) and a total fluorescence change (ΔF). All the parameters were changed as a function of [TFE] and [heparin] (Fig. 2). For example, by increasing heparin concentration under a fixed TFE concentration, t_d was shortened, k_{agg} was accelerated, and ΔF was increased.

Notably, without TFE, heparin even at 100 μg/ml could not induce the fibrillation (asterisks of Fig. 2A: incubation for one week), whereas 0.5 μg/ml or less of heparin was enough to form fibrils in the presence of TFE (open circles of Fig. 2A). Similarly, TFE was ineffective up to 15% in the absence of heparin, whereas 5% or less of TFE induced the IAPP aggregation with a trace amount of heparin (Fig. 2B). These results indicate that the aggregation enhancement in the heparin-TFE mixture cannot be explained by a simple sum of the two independent cofactor's actions. The coexistence effect of the two is non-linearly strong. We do not assert that the synergistic effect is always required for the IAPP aggregation at higher IAPP concentration or at higher cofactors concentrations than those we tested here. Our conclusion was stated within this limit. In

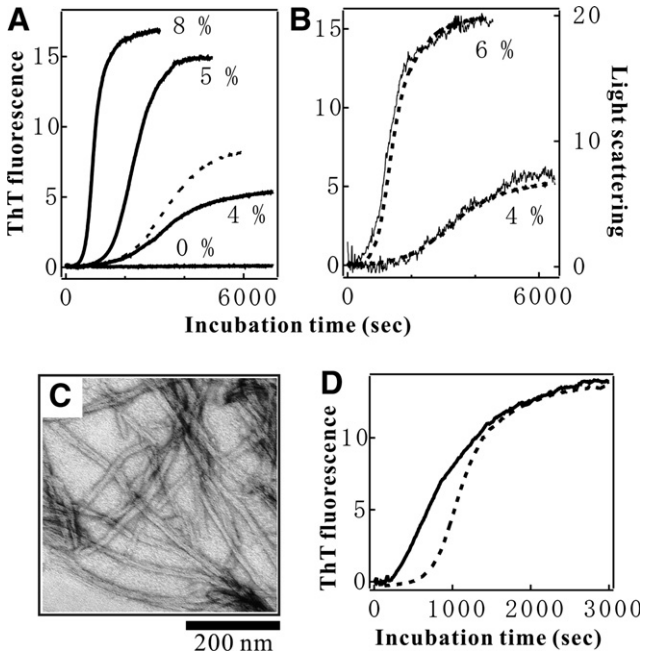

Fig. 1. IAPP aggregation in heparin-TFE mixtures. (A) Aggregation monitored by ThT fluorescence. IAPP at 0.5 μM was incubated at 37 °C in 20 mM Tris–HCl (pH 7.5), 0.1 M NaCl and 5 μM ThT. Solid lines: [heparin] = 5 μg/ml and [TFE] = 0%, 4%, 5% or 8% (w/v). A broken line: [heparin] = 0.5 μg/ml and [TFE] = 5 %. (B) Aggregation monitored by ThT fluorescence (broken lines) and light scattering (thin solid lines). [heparin] = 5 μg/ml and [TFE] = 4% or 6% (w/v). (C) A TEM image of IAPP aggregates formed in 5% TFE and 5 μg/ml heparin incubated for 2 h. (D) A seeding effect on the IAPP aggregation kinetics. A reference run without seeds was done with 0.5 μM IAPP, 5% TFE and 5 μg/ml heparin (a broken line). A seeded run (a solid line) was done in the same condition as that of the reference run except that seeds of fibrils are added. The sample solution after the reference run for 2 h was used as the seeds. The seed volume was 1/20 of the total volume.

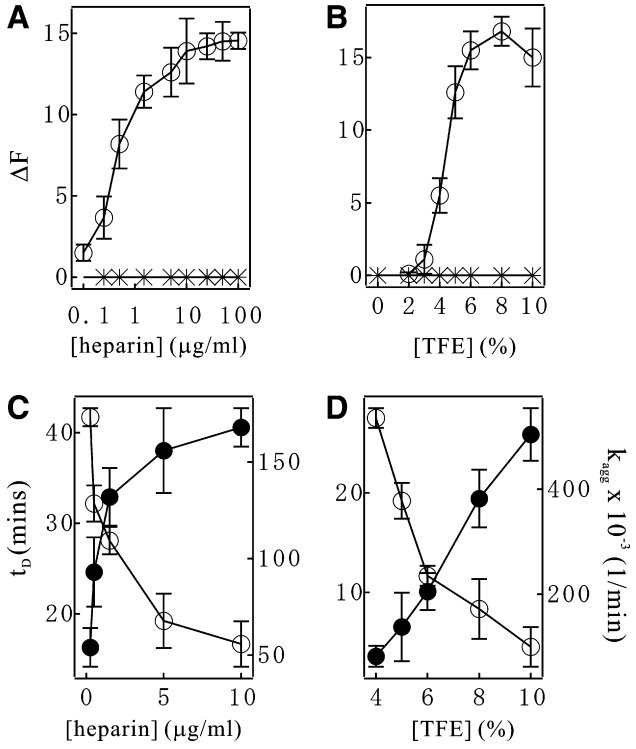

Fig. 2. Kinetic parameters for the IAPP fibrillation monitored by ThT fluorescence. Experimental conditions were the same as those of Fig. 1A. Each kinetic trace was fitted by Eq. (1). (A) ΔF for 0% (asterisks) or 5% TFE (circles) at various heparin concentrations. A log scale was used for the horizontal axis. (B) ΔF for 0 μg/ml (asterisks) or 5 μg/ml heparin (circles) at various TFE concentrations. To obtain the ΔF values for the asterisks in (A) and (B), the sample solutions were incubated at 37 °C for one week. (C) t_d (open circles) and k_{agg} (filled circles) for 5% TFE at various heparin concentrations. (D) t_d (open circles) and k_{agg} (filled circles) for 5 μg/ml heparin at various TFE. Data points of (A)–(D) are given by averaging three or four independent experiments. Error bars are the standard deviations.

fact, polyanions, without assistance of others, are known to enhance the IAPP fibrillation at higher peptide concentrations [11,20].

3.3. Effects of other alcohol and polyanion species

A variety of alcohol species could cooperate with heparin in the IAPP fibrillation (Fig. 3). In the presence of 5 μg/ml heparin, HFIP induced the fibrillation at less than 1 % (Fig. 3A). In case of isopropanol (iPrOH) or ethanol (EtOH), higher alcohol concentrations are required for the fibrillation (Fig. 3B). The order of the efficiency was HFIP > TFE > iPrOH > EtOH (Fig. 3C). We also tested the effects of other polyanion species, *N*-acetylheparin and polyE, which could also yield the IAPP fibrils only when the solution contained TFE (Fig. 4).

3.4. Possible mechanisms of the synergistic actions

Possible mechanisms of the synergistic effect of polyanion and alcohol could be as follows. Alcohol at low concentrations reduces backbone solubility of polypeptide chain and stabilizes hydrogen bonds between peptide components [21]. Polyanion attracts basic amino acid residues and condensates polypeptide chains at the polyanionic sites [11,12]. The alcohol-driven hydrogen bonding within a polypeptide chain combined with the polyanion-mediated neutralization of the intra-chain electrostatic repulsion could generate a compact polypeptide conformation prone to amyloid-type assembly. This mechanistic interpretation of our results is consistent with a finding by Munishkina et al. [16] that the partially folded compact conformation induced by relatively low concentrations of alcohols enhances the fibril formation of α-synuclein. Alternatively, well-ordered amyloid-type assembly of the peptide could also be directed by regular arrangement of the polypeptide chains at the regularly spaced polyanionic sites, where the alcohol effect could further refine and stabilize this peptide assembly by forming inter-molecular hydrogen bonding.

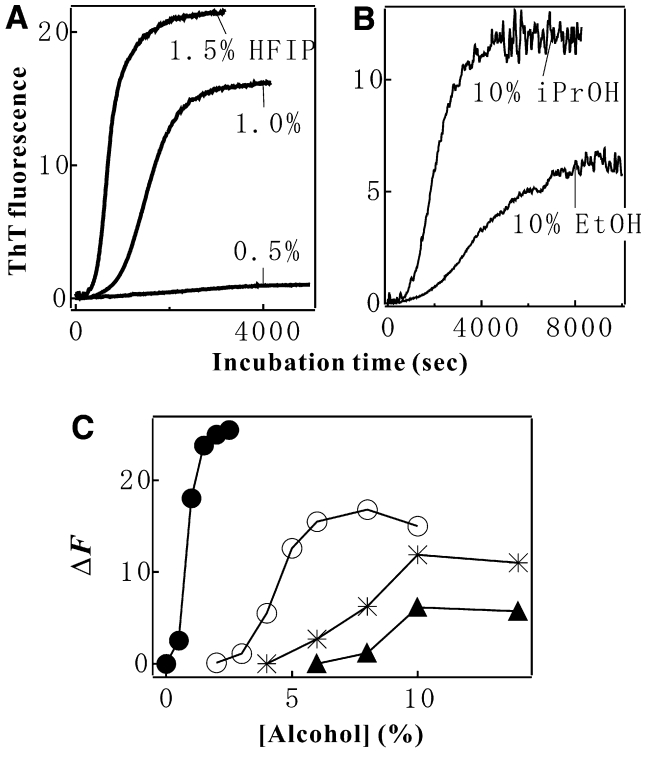

Fig. 3. IAPP aggregation induced by various alcohol species. IAPP of 0.5 μM were incubated at 37 °C in 20 mM Tris–HCl (pH 7.5), 0.1 M NaCl, 5 μM ThT and 5 μg/ml heparin. Aggregation kinetics was monitored by ThT fluorescence. Kinetic traces in the presence of 0.5%, 1% or 1.5% HFIP (A) or of 10% iPrOH or 10% EtOH (B). In panel (C), Δ*F* values are plotted against the concentration of HFIP (filled circles), TFE (open circles), iPrOH (asterisks) or EtOH (triangles).

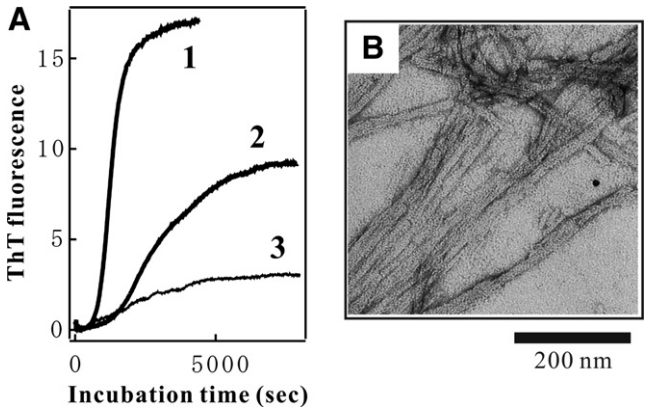

Fig. 4. IAPP aggregation induced by various polyanion species. IAPP of 0.5 μM were incubated at 37 °C in 20 mM Tris–HCl (pH 7.5), 0.1 M NaCl, 5 μM ThT and 6% TFE. (A) Aggregation kinetics monitored by ThT fluorescence in the presence of 5 μg/ml heparin (trace 1), 50 μg/ml *N*-acetylheparin (trace 2) or 200 μg/ml polyE (trace 3). (B) A TEM image of IAPP aggregates induced by 200 μg/ml polyE and 6% TFE, incubated at 37 °C for 2.5 h.

We would also mention recent theoretical studies by Kinoshita and his colleagues, which proposed that translational entropy gain of solvents is a primary driving force for the amyloid-type protein assembly [22]. This theory claims that a mixture of water and solute molecules with a variety of molecular sizes can maximize the translational entropy gain accompanied with the ordered protein assembly [23]. This mechanism of entropic origin might also contribute for the enhancement of the amyloidgenesis in the media comprising a variety of molecular species as observed in the present study.

3.5. Biological implications

Polyanionic molecules such as GAGs and nucleic acids are abundant in human tissues, and have been suggested to promote amyloidgenesis [3,14,15,20]. However, the effects of polyanions must be weak in physiological media containing counter cations. The weakened polyanionic effects in vivo may require synergistic action with the non-polar milieu for promoting the amyloidgenesis. On the other hand, a surface of biomembrane is a plausible site of amyloid nucleation [3,7,8,24], where negative charges at head group of lipid molecules are essential for the fibril formation [10,25]. It means that co-existence of anionic and non-polar parts of the lipid membrane is essential. These considerations agree well with the present observations in the polyanion–alcohol mixtures. Strong synergistic action of multiple cofactors might play important roles in amyloidgenesis in the living body.

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