Islet amyloid polypeptide and high hydrostatic pressure: Towards an understanding of the fibrillization process

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Abstract. Type II Diabetes Mellitus is a disease which is characterized by peripheral insulin resistance coupled with a progressive loss of insulin secretion that is associated with a decrease in pancreatic islet β-cell mass and the deposition of amyloid in the extracellular matrix of β-cells, which lead to islet cell death. The principal component of the islet amyloid is a pancreatic hormone called islet amyloid polypeptide (IAPP). High-pressure coupled with FT-IR, CD, ThT fluorescence spectroscopic and AFM studies were carried out to reveal information on the aggregation pathway as well as the aggregate structure of IAPP. Our data indicate that IAPP pre-formed fibrils exhibit a strong polymorphism with heterogeneous structures very sensitive to high hydrostatic pressure, indicating a high percentage of ionic and hydrophobic interactions being responsible for the stability the IAPP fibrils.

1. Introduction

Amyloid formation and accumulation are implicated in a number of diseases, such Alzheimer’s and Parkinson disease, Type II Diabetes Mellitus (TIIDM) and prion disorders [1, 2], and seem to be the central factors in the development of the symptoms of these diseases. Proteins can form different types of insoluble aggregate. One the one hand, amorphous aggregates may be formed, which have multiple protein conformations with rather ill-defined intermolecular interactions, in contrast to protein crystals that can generally be characterized by a single protein conformation with a well-defined intermolecular packing arrangement. On the other hand, also some ordered non-crystalline protein structures such as amyloid fibrils share these properties. In fact, amyloid can be thought of as a one-dimensional crystal in which packing in the plane perpendicular to the direction of fibrillar growth is not homogeneous [3]. Amorphous aggregates generally form rapidly when the protein concentration exceeds the solubility limit. Conversely, protein crystal formation requires time, owing to the kinetic barrier imposed by nucleus formation, which is the rate determinant step. Ordered non-crystalline protein polymer structures such as amyloid share this requirement for nucleation. Nucleus formation requires a series of association steps that are thermodynamically unfavourable (equilibrium constant for nucleation $K_n \ll 1$) because the resultant intermolecular interactions barely compensate the entropic costs of association. Once the nucleus has formed, further addition of monomers becomes thermodynamically favourable (equilibrium constant for growth process $K_g \gg 1$), however, because the monomers contact the growing polymer at multiple sites, resulting in rapid polymerization-like growth.

A key step in the fibrillation process is the transition of a protein from its native structure to a β-sheet arrangement [1,3,4]. Upon formation of amyloid fibrils, the protein molecules that compose the fibrils (partially) lose their native conformation and generally adopt ordered, stacked cross-β-sheet structures. These observations suggest that prevention of the ability of amyloidogenic proteins to nucleate or later, adopt a β-sheet conformation would be useful as a way to interfere with the amyloid self-assembly process. Despite the recent advances in the theoretical and experimental techniques to understand these aggregation processes, the underlying mechanisms have proved challenging to study. This is essentially due to the irreversibility of protein aggregation under ambient temperature and...
pressure conditions, which complicates or even prohibits the analysis of the underlying kinetic, thermodynamic, and structural parameters.

IAPP is a 37 amino acid residue peptide hormone [5] that is co-synthesized and co-secreted with insulin by pancreatic β-cells [6]. Several functions have been associated with the soluble form of this hormone [6-10], including the control of hyperglycemia by restraining the rate at which dietary glucose enters the bloodstream. For reasons that are still not fully understood, IAPP aggregates in the extra cellular matrix of the β-cells forming fibrillar amyloid deposits. These deposits are present in approximately 95 % of TIIIDM patients and are strongly associated with islet β-cell degeneration and loss [11,12].

It has been proposed that the IAPP aggregation process has two distinct phases: a lateral growth of oligomers followed by longitudinal growth into mature fibrils [13-15], and it has been demonstrated that the initial stages of IAPP fibril formation are driven by the increase of solvent-exposure of hydrophobicity patches [13]. The structural changes behind the fibrillization process are still poorly understood, however.

High hydrostatic pressure (HHP) has been widely used as a tool to understanding protein folding [16-22]. High pressure tends to destabilize proteins due the fact that the protein-solvent system in the unfolded state occupies a small volume than the system in the native state. In a similar way, pressure leads to the dissociation of oligomeric proteins. These effects seem to be caused by a combination of factors. The presence of cavities within the folded proteins or in the interface of oligomers favour unfolding or dissociation of these structures [16,17]. The dissociation of electrostatic interactions also leads to a marked reduction in the overall volume caused by electrostrictive effects of the water molecules around the unpaired charged residues [23]. In a similar way, solvation of polar groups results in a decrease in volume. These effects compensate for the increase in volume as the crystalline-like state of the protein interior is disrupted, exposed to solvent and hydrated upon unfolding.

The use of the pressure in studies of protein folding and stability can offer a great number of advantages [16,17]. Pressure affects only the volume of the system in focus, while the temperature denaturation, for example, involves changes in both volume and thermal energy of the system. Also, temperature-induced aggregation generally results in an irreversible aggregation process, which may be explained by less disfavoured hydrophobic interactions at higher temperatures. On the contrary, denaturation by high pressure generally is a reversible process. Furthermore, high pressure treatment can lead to dissociation of aggregated structures and may result in formation of monomers and natively folded structures. Also, measurement of the activation volume for pressure-induced kinetic folding or unfolding reactions is one of the few methods yielding structural information regarding the transition states [24]. Hence, in addition to co-solvent and temperature perturbation, pressure dependent studies shed new light on alternative folding/aggregation pathways and their intermediate states.

So far, there are no reports regarding the effects of HHP on IAPP. Owing to the fact that high hydrostatic pressure acts to disfavour hydrophobic and electrostatic interactions that cause protein aggregation, this parameter can be used as efficient tool to reveal new important information on the nucleation and growth processes of the protein. High-pressure coupled with Fourier-transform Infrared (FT-IR) spectroscopic studies and atomic force microscopy (AFM) measurements complemented by Thioflavin T (ThT) fluorescence and circular dichroism (CD) experiments were carried out to reveal the changes in IAPP aggregate and fibril formation under pressure-perturbation. These results lead to a better understanding of the aggregation pathways and the possible amyloidogenic states of IAPP and may hence contribute to a better understanding of the pathogenesis of type II diabetes mellitus. Moreover, the results obtained may prove useful for the identification and molecular characterization of toxic intermediate states that may be used as targets for parallel searching of compounds that can interfere with their formation.
2. Materials and Methods

Synthetic human IAPP was obtained from Calbiochem-Novabiochem (Bad Soden, Germany). NaH₂PO₄ buffer was purchased from Gibco BRL, 2,2,2-trifluoroethanol (TFE) was obtained from Aldrich. ThT, D₂O and all other reagents were from Sigma. The reagents were of the highest analytical grade available. Pre-distilled water was filtered and deionised through a Millipore purification system.

Incubation under pressure was carried out in a pressure cell equipped with sapphire optical windows, similar to that originally described by Paladini and Weber [25,26]. The temperature of the pressure cell was controlled by means of a jacket connected to a circulating bath. Pressure was increased stepwise in increments of 150 bar until 3.5 kbar. The windows of the pressure cell were flushed with nitrogen at low temperatures to prevent water condensation.

IAPP aggregation and fibril formation was determined by CD measurements using a Jasco J-720 spectropolarimeter. CD spectra were collected over the wavelength range from 190 to 260 nm at 0.2 nm intervals with a spectral bandwidth of 0.8 nm and 1 s integration time. The cell holder was maintained at 25 ± 0.1 °C. For the CD measurements, IAPP stock solutions were kept in 100 % TFE at 4 °C and diluted prior to the experiments to give the chosen final concentration in 10 mM NaH₂PO₄ (pH 7.4), with residual 1 % TFE. CD spectra, taken at least four times, are presented after subtracting the spectra of buffer and smoothing, using the Origin 7 scientific graphic and analysis software. The data are expressed as molar ellipticity $[\theta]$ in units of deg cm² dmol⁻¹.

ThT spectroscopic measurements were carried out using an ISS K2 multifrequency phase fluorometer (ISS Inc., Champaign, IL) coupled with a water circulating bath to keep the chosen temperature constant. The samples were prepared as for the CD measurements and ThT was added to a final concentration 8 times higher than that of the protein. The fluorescence signal (excitation at 450 nm) was recorded between 450 and 600 nm using 1 nm slits both for the emission and excitation measurements.

For the FT-IR measurements, CaF₂ transmission windows with 0.05 mm Teflon spacers were used. The temperature in the cell was controlled through an external water-circuit. FT-IR spectroscopy has proven to be a powerful technique to determine the secondary structure elements [17,27-29]. The amide I' band (between 1600 and 1700 cm⁻¹) was recorded, which is mainly associated with the carbonyl stretching vibration of the amide groups and which is directly related to the backbone conformation and hydrogen bonding pattern of the protein [29]. For scanning the samples before and after pressure treatment, we used 10 mM NaH₂PO₄ with 1 % residual TFE in D₂O at pD 7.4 and a protein concentration of 250 μM (~0.1 % w/w). The FT-IR spectra were collected on a Nicolet 5700 FT-IR spectrometer equipped with a liquid nitrogen–cooled MCT detector. For each spectrum, 256 interferograms of 2 cm⁻¹ resolution were co-added. The sample chamber was continuously purged with dry air. From the spectrum of each sample, a corresponding buffer spectrum was subtracted. All the spectra were baseline-corrected and normalized for the amide I' band area. Data processing was performed with GRAMS software.

For the atomic force microscopy (AFM) measurements, samples were diluted with deionised water to yield a final concentration of 1 μM. 30 μL were applied onto freshly cleaved muscovite mica and allowed to dry. Data were acquired in the tapping mode on a Multi Mode TM SPM AFM microscope equipped with a Nanoscope IIIa Controller from Digital Instruments. As AFM probes, Silicon SPM Sensors "NCHR" (force constant, 42 N/m; length, 125 mm; resonance frequency, 300 kHz) from Nanosensors were used [30,31].

3. Results

In order to investigate the stability of IAPP towards high pressure, either fresh peptide or pre-formed fibrils were subjected to pressures up to 3.5 kbar and the changes were monitored by different techniques to yield information about the transformation process and the structures evolving at various levels of complexity. The protein solutions were prepared from stock solutions in a water buffer with 1 % residual TFE. Such condition has been shown to give raise to or to synchronize the formation of pre-assembled β-sheet structures which are rich in fibrils and protofibrils [32].
The first step was to investigate the ability of IAPP to bind ThT – a fluorophore, widely used to detect amyloid structure in proteins [33] – under different pressure conditions. First, ThT analysis (ratio IAPP/ThT 1:8) of the amyloid content was performed 5 min after hydration of the protein (figure 1). The sample showed a substantial emission at 482 nm, characteristic of ThT bound to IAPP amyloid [34], already after such a short time, in agreement with previous studies on IAPP [35]. Then, the sample was split into two aliquots, one kept at ambient pressure and the other was subjected to 3.5 kbar for three subsequent days. After this incubation period, both samples were checked again for ThT fluorescence emission. The IAPP sample aggregated for three days at ambient pressure was not able to bind further ThT, and almost no fluorescence was detected which is due to the fact that the aggregate precipitated. However, when the sample was subjected to pressures of 3.5 kbar for 3 days, further binding of ThT was observed (figure 1). Under these conditions, the amyloid is probably partially disrupted and smaller species, such as IAPP oligomers and protofibrils, which can bind ThT [36], are formed being able now to bind more ThT. As mature fibrils are normally very resistant even to extremes of high pressure [17,33], we might conclude that these IAPP aggregates formed at ambient pressure conditions do not consist (only) of mature, densely packed "cores", but contain a significant amount of species which are sensitive to HHP.

In a second step, we were looking for corresponding changes in the secondary structure of the samples. For this purpose, far-UV CD spectroscopy has been used, which is a sensitive technique that allows assessing the secondary and tertiary structure of proteins in solution [37-39] and hence permits evaluating changes in the conformation of the IAPP before and after pressure treatment. A line shape analysis of the CD spectra can be used for distinguishing early conformational events that precede fibril formation. As for the ThT measurements, we analyzed the secondary structure content of the sample 5 min after sample preparation. As shown in figure 2, formation of a typical β-sheet conformational transition state is clearly visible. Calculation of the secondary structure content [38,39] from the CD spectra of this solution using the protein reference spectra by Srerama et al. [40] suggests that IAPP at early stages of aggregation at 25 ºC still contains substantial amounts of random
coil (64.1%), 20.2% of β-sheets and turns and some α-helical structures (~15.7%). These data are in reasonable agreement with previous reports for soluble IAPP by Kayed et al. [13] (random coil: 71%, β-sheets and turns: 21%, α-helices: 8%). CD spectra recorded after 3 days at room temperature and ambient pressure show a decrease in the α-helical content and a concomitant increase in β-sheet structures indicated by an increase in of modulus of the ellipticity at ~220 nm, which is due to increasing aggregate formation. On the other hand, the CD spectrum of the pressurized IAPP sample looks different, with a similar minimum but shifted to about 222 nm, however the overall CD ellipticity has decreased drastically, most likely due to precipitation of the IAPP aggregate (as can be seen by light scattering).

In a third step we were looking deeper into the secondary conformational changes using FT-IR spectroscopy, a common tool used for monitoring α-helix to β-sheet transitions and to disentangle between different β-sheet structures that accompany protein aggregation processes [27,33,41], and which can also be used for pressure-dependent spectroscopic studies. Figure 4 shows FT-IR spectra of various IAPP samples: freshly dissolved IAPP and IAPP aggregated for 3 days at ambient pressure and at 3.5 kbar, respectively (all spectra were taken at room temperature, 25 °C). The FT-IR spectrum of the freshly prepared IAPP sample at a concentration as high as 250 μM exhibits a IR band appearing at ~1622 cm⁻¹, likely of intermolecular parallel β-sheet structures, and a pronounced peak at 1673 cm⁻¹, which is due to turns and residual trifluoracetic acid (TFA) in the sample. Such IR pattern is indicative of the aggregated state of IAPP according to data of other amyloidogenic proteins, like insulin [41]. After the incubation period of 3 days at ambient pressure and temperature, the infrared spectrum shows similar features, with a broadening and a small shift of the amide I' band to slightly smaller wavenumbers (~1618 cm⁻¹), however. Very likely, a distribution of different sizes and shapes of IAPP aggregate is found initially, and the overall size of the structures evolving increases with

Figure 2. IAPP amyloid formation followed by CD spectroscopy. Far-UV CD spectra of IAPP solutions (10 μM in 10 mM NaH₂PO₄ buffer (pH 7.4) containing 1% TFE, T = 25 °C) subjected to different treatments: (■) sample at ambient pressure after 1 d, (○) sample at ambient pressure after 3 d (precipitation occurs), (Δ) sample pressurized at 3.5 kbar for after 3 d. Non-specific background was subtracted from the samples by using appropriate blanks not containing the protein.

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increasing IAPP concentration. After the incubation period it seems that the β-strands are realigning from a non-perfectly packed initial state to more ordered structures with stronger H-bonding, leading to the observed shift of the amide I' band to 1618 cm⁻¹ accompanied by an increase of the β-turn content.

**Figure 3.** FT-IR spectra (at the top of the figure) of IAPP: (--) 5 min after preparation, (-----) after 3 d of aggregation in 10 mM NaH₂PO₄ (pD 7.4) at ambient pressure, (---) after 3 d at 3.5 bar. All data were taken at \( T = 25 \) °C. At the bottom of the figure, also the second derivative spectra are shown, which allow a more accurate determination of peak positions.

When IAPP was aggregated under pressure, the main amide I' IR band shifts to significantly smaller wavenumbers (≈1614 cm⁻¹), indicating that the pressure treatment leads to the formation of a different aggregate structure, probably with stronger intermolecular H-bonding between β-sheet strands, or to a larger population of these species.

In order to reveal the different morphological structures formed, additional AFM measurements were carried out for this IAPP concentration (figure 4). Most of the aggregate structures seen in the sample that was not subjected to HHP are short (< 1 μm) fibrils with an average of 5 - 15 nm diameters as determined from the AFM height profile. Such rather short fibrillar structures probably appear due to a comparably fast nucleation process at this high protein concentration, whereas the elongation process seems to be comparably slow. The pressure treated sample still contains fibrils, but also a significant amount of smaller oligomeric particles (of 0.5 - 1.5 nm size).
Figure 4. AFM height images of 250 μM IAPP aggregates after 3 d without (panel A) and with pressure treatment at 3.5 kbar (panel B). The same samples analyzed by FT-IR and CD spectroscopy were diluted with deionized water to yield a final concentration of 1 μM. 30 μL were applied onto freshly cleaved muscovite mica and allowed to dry before the AFM analysis.
Taking the FT-IR spectroscopic and AFM results together, we may conclude that the sample subjected to high pressure treatment displays less fibrillar β-sheet structures and a larger population of smaller amorphous aggregates with a different H-bonding pattern. To support these conclusions, the samples without and with 3 d pressure treatment were centrifuged at 16,000 rpm at 4 °C for 20 min in order to remove any insoluble material and the protein concentration of the supernatant was determined from the UV absorbance at 274.5 nm, using a molar extinction coefficient of 1440 M⁻¹cm⁻¹ [13]. Protein was detected only for the pressurized IAPP (~17 % of the overall protein concentration). Immediately after the absorbance measurements, also far-UV CD spectra were taken. The HHP treated sample showed a strong negative band at about 200 nm and a positive band at 230 nm, characteristic for the presence of unordered structures (data not show).

The different fibrillar and non-fibrillar amorphous/oligomeric morphologies found for the high pressure treated sample indicate that not all IAPP aggregate structures are equally sensitive to pressure, hence suggesting the existence of pressure resistant fibrils with densely packed cores and a population that can be dissociated by HHP.

4. Conclusions
Here we report on the first high pressure work on IAPP aiming to reveal new information on its aggregation pathways and aggregate/fibril structures. The conceptual framework for using such pressure axis experiments is as follows: The interior of proteins is largely composed of rather efficiently packed residues (with void volume on the order of 0.5 %), more likely hydrophobic than those at the surface. High hydrostatic pressure induces conformational fluctuations due to a decrease in the strength of hydrophobic interactions, finally leading to partially pressure-induced unfolding through transfer of water molecules into the protein interior, gradually filling cavities and leading to the dissociation of close hydrophobic contacts and subsequent swelling of the hydrophobic protein interior [28,42]. According to the literature, aggregation of the C-terminal domain of IAPP (amino acid sequences 20-29 and 30-37) is thought to be most likely driven by hydrophobic interactions. The distinctly amyloidogenic region 20-29 has been pointed out to be a key fibril-forming region [43]. Hence it is conceivable that the C-terminus of IAPP, spanning residues 20 to 37, forms a contiguous β-sheet. The secondary structure predictions of human IAPP indicate that there is one potential α-helical region between amino acid residues 8-14, and three potential β-strand regions. A β-turn has been predicted at Asn31, which would result in two adjacent β-strands (32-37 and 24-29); a third β-strand is proposed to exist in region 18-23 [44]. According to these data, it may be concluded that IAPP has not only one but several amyloidogenic cores that are interacting to form an organized aggregate structure, and that hydrophobic interactions may drive the initial stage of the aggregation process. As HHP is acting to weaken or even prevent hydrophobic self-organization, we may expect that the amyloidogenic "cores" cannot be arranged and packed the same way upon aggregation as they are able to do under ambient pressure conditions, thus leading to formation of a more heterogeneous population of fibrils and smaller amorphous/oligomeric species.

Taken all data together, a hypothetical model for IAPP fibril formation may be suggested: i) IAPP undergoes fast nucleation (due to several amyloidogenic "cores"), largely driven by hydrophobic interactions. Hence, formation and packing of fibrils is not perfect and mixed-registry β-sheet structures might exist, in particular at high protein concentrations, which can partially be dissociated by pressure leading to smaller aggregate structures and oligomers. HHP already of pressures as low as 3.5 kbar is sufficient to weaken and (at least partially) disrupt the hydrophobic cores thus leading to formation of a heterogeneous population of fibrillar aggregates with IR amide I’ bands in the low wave number region (which is typical of a more strongly H-bonding pattern of intermolecular β-sheets) and a large amount of non-fibrillar smaller aggregates and oligomers, as detected by AFM (with IR amide I’ bands in the larger wave number region around 1620 cm⁻¹). ii) Our data also indicate that the pre-formed IAPP fibrils are sensitive to high hydrostatic pressure, similar to amorphous aggregates and inclusion bodies [45-47]. Considering the fact that high hydrostatic pressure is an effective means in disturbing ionic and hydrophobic interactions but not hydrogen bonds, we can conclude that these
former two types of interaction are important for the stability of IAPP fibrillar aggregates, as also suggested in work using denaturing agents [13]. The results may be physiologically relevant and may have important implications regarding the stability of the IAPP fibrils against degradation, a fact that can contribute to the development of antagonists of the pathogenesis of type II diabetes mellitus.

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