The role of the disulfide bond in the interaction of islet amyloid polypeptide with membranes

Lucie Khemtémourian · Maarten F. M. Engel · John A. W. Kruijtzer · Jo W. M. Höppener · Rob M. J. Liskamp · J. Antoinette Killian

Abstract Human islet amyloid polypeptide (hIAPP) forms amyloid fibrils in pancreatic islets of patients with type 2 diabetes mellitus. It has been suggested that the N-terminal part, which contains a conserved intramolecular disulfide bond between residues 2 and 7, interacts with membranes, ultimately leading to membrane damage and β-cell death. Here, we used variants of the hIAPP1–19 fragment and model membranes of phosphatidylcholine and phosphatidylserine (7:3, molar ratio) to examine the role of this disulfide in membrane interactions. We found that the disulfide bond has a minor effect on membrane insertion properties and peptide conformational behavior, as studied by monolayer techniques, 2H NMR, ThT-fluorescence, membrane leakage, and CD spectroscopy. The results suggest that the disulfide bond does not play a significant role in hIAPP-membrane interactions. Hence, the fact that this bond is conserved is most likely related exclusively to the biological activity of IAPP as a hormone.

Keywords Amylin · Protein–membrane interactions · Model membranes · 2H NMR · Circular dichroism · Type II diabetes mellitus

Introduction

Type 2 diabetes mellitus (DM2) is characterized histopathologically by the presence of fibrillar amyloid deposits in the pancreatic islets of Langerhans (islet amyloid). The main component of these amyloid fibrils is the 37-amino-acid residue human islet amyloid polypeptide (hIAPP), a hormone that is normally co-secreted with insulin by the β-cells of the pancreas (Westermark et al. 1987). Although the normal physiological role of hIAPP is not entirely clear, it has been shown that the peptide plays a role as a hormone in suppression of gastric emptying, food intake, and glucose homeostasis (Akesson et al. 2003; Reda et al. 2002; Rushing et al. 2001).

It is thought that hIAPP amyloid formation is cytotoxic to the islet β-cells (Höppener et al. 2000; Khemtémourian et al. 2008), and that this cytotoxicity involves membrane disruption by growing IAPP fibrils (Engel et al. 2008) or by toxic IAPP oligomers (Demuro et al. 2005; Engel 2009; Jayasinghe and Langen 2007; Kayed et al. 2003). It is well established that hIAPP is able to interact with membranes and that membranes can promote the aggregation of hIAPP (Knight and Miranker 2004; Janson et al. 1999; Sparr et al. 2002).
The initial step of membrane interaction of mature hIAPP is insertion of its N-terminus, most likely as the monomer (Engel et al. 2006). This N-terminus contains an evolutionary conserved disulfide bond between Cys2 and Cys7, presumably formed in the oxidizing environment of the endoplasmic reticulum. This disulfide bond has been shown to be essential for biological activity of hIAPP, i.e. its effect on insulin-stimulated glycogen synthesis (Roberts et al. 1989). Possibly related to this, an important role for the disulfide bridge was demonstrated in heparin binding (Abedini et al. 2006). With regard to its role in amyloid formation, it has been shown that the disulfide prohibits the N-terminal region of hIAPP from adopting a β-sheet-rich structure (characteristic of amyloid fibrils) and that it is involved in amyloid formation in solution, albeit not as part of the core (Koo and Miranker 2005). However, to our knowledge no reports have yet been published on the possible role of the disulfide bond of hIAPP in membrane-interaction.

Because the disulfide bond is located in the N-terminal part of the peptide, and because the N-terminal part is primarily responsible for the interaction of hIAPP with membranes, we examined the effect of this disulfide on insertion of the peptide hIAPP_{1-19} into membranes. In particular, we have examined the aggregational behavior, the membrane insertion, the secondary structure and the effect on lipid acyl chain order and on membrane leakage.

Preparation of peptide samples

Peptide stock solutions were prepared by dissolving the peptide at a concentration of 1 mM in hexafluoroisopropanol (HFIP) and incubating for at least 1 h. Next, HFIP was evaporated, followed by vacuum desiccation for 20 min. The resulting peptide film was then dissolved to a final peptide concentration of 1 mM in either DMSO for the monolayer or in an appropriate buffer containing the vesicles for the CD and the NMR experiments.

Monolayer experiments

Peptide-induced changes in the surface pressure of a monomolecular layer of phospholipids at constant surface area were measured with the Wilhelmy plate method, as reported previously (Engel et al. 2006). Surface pressures were measured at 22(±2)°C. Briefly, a Teflon trough was filled with 6.0 ml 10 mM Tris–HCl, 100 mM NaCl buffer (pH 7.4). Lipid monolayers were spread from a 1 mM stock solution in chloroform, after which 10 μl of a 600 μM freshly prepared stock solution of the peptide was injected into the sub-phase. The final peptide concentration was 1 μM.

Preparation of LUVs (large unilamellar vesicles)

LUVs were prepared from POPC/POPS in a 7:3 molar ratio. Stock solutions of POPC and POPS in chloroform at concentrations of 20–30 mM were mixed in a 7:3 molar ratio in a glass tube. The solvent was evaporated with dry nitrogen gas yielding a lipid film that was subsequently kept in a vacuum desiccator for 20 min. Lipid films were hydrated in 10 mM phosphate buffer (pH 7.4) for at least 24 h.

Materials and methods

Materials

1-Palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC), 1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-L-serine (POPS), and chain perdeuterated POPC and POPS (POPC-2H_{31} and POPS-2H_{31}) were purchased from Avanti Polar Lipids (Alabaster, AL, USA).

Peptide synthesis and purification

The peptides (see Fig. 1 for the amino acid sequence) were synthesized using Fmoc chemistry on a Tentagel S RAM resin to provide an amidated C-terminus. Fmoc–Cys(Me)–OH was used for the synthesis of the protected peptide; Fmoc–Cys(Trt)–OH was used for the synthesis of the linear peptide. Linear hIAPP_{1-19} was dissolved in aqueous DMSO and oxidized with air to the corresponding disulfide bond. All peptides were purified by HPLC using an Altima C8 column (100 Å pore size, 10 μm particle size, 25 × 2.2 cm). The peptides were eluted with a flow rate of 12.5 ml/min. using a linear gradient of buffer B (100% in 100 min) from 100% buffer A (0.1% TFA in CH_{3}CN–H_{2}O 5:95 v/v), buffer B (0.1% TFA in CH_{3}CN–H_{2}O 95:5 v/v). Purity of the peptides was higher than 95% as determined by analytical HPLC, and the masses of the peptides were confirmed with MALDI-TOF mass spectrometry.

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30 min, at a lipid concentration of 10 mM. The lipid suspensions were subjected to ten freeze–thaw cycles, at temperatures of approximately –80 and 40°C, and subsequently extruded ten times through 0.2 µm pore-size filters (Anotop 10; Whatman, Maidstone, UK).

Results and discussion

To evaluate the role of the disulfide bond in hIAPP₁₋₁₉ on its membrane interactions, we synthesized two hIAPP₁₋₁₉ peptides: an oxidized form and a protected form with a methyl group on each cysteine residue to avoid the formation of the disulfide bond during the experiments. The amino acid sequences of the peptides are shown in Fig. 1.

The oxidized peptide has a slightly higher tendency to insert than the protected peptide

To study the insertion of the peptides in lipid membranes, we performed monolayer experiments. Injection of a solution of either oxidized or protected hIAPP₁₋₁₉ into the aqueous subphase below a POPC/POPS (7:3) monolayer resulted in a fast increase in the surface pressure after addition of each peptide. After 20 min, at an initial surface pressure of 24 mN/m, the increase in surface pressure induced by insertion of the oxidized hIAPP₁₋₁₉ was 9.0 ± 0.5 mN/m, compared with an increase of 7.5 ± 0.5 mN/m for the protected hIAPP₁₋₁₉ (Fig. 2a), indicating efficient insertion for both peptides.

Next, we determined the maximal initial surface pressure at which the peptides were still able to insert, i.e. cause a further increase in surface pressure, by analysing the surface pressure increase as a function of initial surface pressure. The data from Fig. 2b indicate that the extrapolated “limiting surface pressure” is high for all peptides and it seems to be slightly higher for the oxidized peptide (47.2 ± 0.7 mN/m) than for the protected peptide (44.7 ± 0.8 mN/m). In both cases, the limiting surface pressure is significantly higher than the surface pressures that correspond to the packing density in lipids in biological membranes, between 31 and 35 mN/m (Demel et al. 1975), indicating that in vivo also hIAPP₁₋₁₉ can insert efficiently into membranes. The limiting surface pressure of hIAPP is of the same order, or even higher, than that of peptides with a known membrane activity, for example neuropeptides and antimicrobial peptides (Calvez et al. 1975). Thus, our results suggest that both peptides insert very efficiently into membranes, but that the oxidized peptide has a slightly higher capacity to insert than the protected peptide.

The oxidized peptide induces slightly more acyl chain disorder than the protected peptide

Next we investigated the effect of membrane insertion of these peptides on lipid chain order by ²H NMR. The peptides were incorporated into bilayers of perdeuterated POPC/POPS at a 1:20 peptide-to-lipid molar ratio. ³¹P NMR measurements confirmed that the lipids in these
systems are in a bilayer organization (data not shown). Figure 3a presents the $^2$H NMR spectra of POPC-$^2$H$_{31}$/POPS bilayer dispersions, with and without the peptides at 25°C. In the case of POPC-$^2$H$_{31}$/POPS, we observe the typical behavior of this system, characteristic of a lamellar fluid phase (Salnikov et al. 2009). The spectra exhibit many resolved splittings. The smallest splitting, corresponding to the methyl groups at the end of the acyl chains, is about 5.0 kHz and the largest, corresponding to the CD$_2$ groups closest to the glycerol backbone, is approximately 25.9 kHz. In the presence of the peptides, no characteristic changes in spectrum shape occur. Thus, addition of the oxidized peptide or the protected peptide does not induce a global perturbation of the dynamics of the membrane matrix. However, the width of the spectra seems slightly reduced, with the largest splitting now being 24.9 kHz for the protected peptide and 24.1 kHz for the oxidized peptide. Figure 3b shows the profile of the order parameter ($S_{CD}$) for each carbon position in the lipid chain in the presence and in the absence of peptide. The POPC-$^2$H$_{31}$ chain order is lower when either peptide is present in the system. This is particularly true for the “plateau” positions (carbons 2 to 6). Toward the chain end (positions 15 and 16) the effect is less marked or absent. In addition, our results show that the POPC-$^2$H$_{31}$ chain order is lower with the oxidized peptide than with the protected one. The $^2$H NMR spectra of POPC/POPS-$^2$H$_{31}$ bilayer dispersions were also recorded. The same effect as for the POPC-$^2$H$_{31}$/
POPS bilayer was observed. The membrane-disorder induced by hIAPP is of the same order as that of peptides with a known membrane activity, for example antimicrobial peptides (Salnikov et al. 2009; Balla et al. 2004). In addition, it was recently shown that Ap42, the peptide that forms amyloid fibrils in Alzheimer’s disease, also induced membrane disorder in POPC/POPS bilayers (Gehman et al. 2008; Lau et al. 2006). This result can be correlated with that of the monolayer study: the oxidized peptide inserts better and induces more disorder into the membrane than the protected peptide, suggesting that the disulfide bond somewhat affects the mode of membrane insertion of hIAPP1–19.

The oxidized peptide has slightly less $\alpha$-helical structure than the protected peptide in the presence of membranes

The small differences in mode of insertion of the oxidized and protected peptide may be because of differences in conformation. We therefore used CD spectroscopy to determine whether the disulfide bridge affects the membrane-bound secondary structure of the peptide in the presence of POPC/POPS membranes. A previous study showed that oxidized hIAPP1–19 adopts $\alpha$-helical structure upon addition of POPG liposomes (Brender et al. 2008). Consistent with this, our CD spectra in POPC/POPS (7:3) of the oxidized peptide display negative ellipticity at 208 and 222 nm, characteristic of $\alpha$-helical backbone structure (Fig. 4). A similar spectrum is observed for the protected peptide. However, the minimum of the protected peptide is slightly more intense than the minimum of the oxidized peptide. This suggests that the protected peptide has a slightly larger $\alpha$-helical content than the oxidized peptide.

This is supported by deconvolution of CD traces, where 62% $\alpha$-helical contribution is obtained for the protected peptide and 58% $\alpha$-helical for the oxidized peptide (inset of Fig. 4). This result suggests that in the presence of membranes the disulfide bond only slightly affects the secondary structure of hIAPP1–19.

The disulfide bond does not influence the effect of hIAPP1–19 on membrane barrier properties or its inability to form amyloid fibers

We wanted to know if the disulfide has an effect on membrane damage and fibril formation. Membrane damage was assayed quantitatively by analyzing the extent of leakage of a fluorescent dye (calcein) from LUVs. The results demonstrate that neither peptide is able to induce significant membrane damage (leakage < 5%, data not shown), despite their ability to efficiently insert into a lipid monolayer and to induce slight bilayer disorder. Also, under these conditions, i.e. in the presence of POPC/POPS LUVs, neither peptide forms fibrils as determined by transmission electron microscopy and thioflavin T fluorescence, even after 15 days of incubation (data not shown). These findings are in agreement with our previous work, which indicated that fibril growth is a requirement for amyloid-induced membrane damage (Engel et al. 2008).

**Conclusion**

Our results suggest that the presence of the disulfide bond has only small effects on membrane interaction of hIAPP. Its removal in hIAPP1–19 results in a slight decrease in membrane insertion, a decrease in the effect of the peptide on membrane disorder, and a small increase in $\alpha$-helical structure. The inability of hIAPP1–19 to induce membrane damage and to form fibrils in the presence of membranes is not changed upon removing the disulfide bond.

Based on the results presented here, and on our previous results (Engel et al. 2006), we conclude that the N-terminal residues of IAPP are important for membrane insertion, but that the disulfide linked residues 2–7 seem much less responsible than the other residues (8–19) for this interaction. This notion is in agreement with two recent studies, which indicate that residues 9 to 22 of hIAPP form a helix that binds to phospholipid bilayers (Apostolidou et al. 2008; Nanga et al. 2009) whereas residues 1 to 8, containing the disulfide, do not interact directly with the membrane. Thus, the fact that the cysteines are evolutionarily conserved is most likely related exclusively to the biological activity of IAPP as a hormone, where these cysteines may be involved in interactions with other cellular components than lipids, e.g. with an IAPP receptor.
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