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Evolutionary Adaptation and Amyloid Formation: Does the Reduced Amyloidogenicity and Cytotoxicity of Ursine Amylin Contribute to the Metabolic Adaption of Bears and Polar Bears?

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Abstract

Much of our knowledge of diabetes is derived from studies of rodent models. An alternative approach explores evolutionary solutions to physiological stress by studying organisms that face challenging metabolic environments. Polar bears eat an enormously lipid-rich diet without deleterious metabolic consequences. In contrast, transgenic rodents expressing the human neuropancreatic polypeptide hormone amylin develop hyperglycemia and extensive pancreatic islet amyloid when fed a high fat diet. The process of islet amyloid formation by human amylin contributes to β -cell dysfunction and loss of β -cell mass in type-2 diabetes. We show that ursine amylin is considerably less amyloidogenic and less toxic to β -cells than human amylin, consistent with the hypothesis that part of the adaptation of bears to metabolic challenges might include protection from islet amyloidosis-induced β -cell toxicity. Ursine and human amylin differ at four locations: H18R, S20G, F23L, and S29P. These are interesting from a biophysical perspective since the S20G mutation accelerates amyloid formation but the H18R slows it. An H18RS20G double mutant of human amylin behaves similarly to the H18R mutant, indicating that the substitution at position 18 dominates the S20G replacement. These data suggest one possible mechanism underpinning the protection of bears against metabolic challenges and provide insight into the design of soluble analogs of human amylin.

Table of Contents

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1. Introduction

Obesity and diabetes have reached epidemic proportions and are an increasing threat to both the developing and developed world. Worldwide, more than 390 million people were estimated to suffer from diabetes in 2013 and the number is projected to increase to over 590 million by 2035.^[1–2] More than 650 million individuals currently suffer from obesity, placing them at risk for type-2 diabetes and heart disease.^[3] These worldwide epidemics necessitate new approaches to the study of diabetes and obesity. Much of our knowledge of diabetes and its complications have been derived from studies of rodent models, but rodent physiology, as related to the regulation of glucose metabolism, is significantly different from human physiology.^[4] An alternative approach is to explore evolutionary solutions to physiological stress through studies of organisms that face metabolic challenges. Recent studies have focused on the adaptations bears make to avoid diabetes, metabolic syndrome and heart disease.^[5–9] Bears face unique metabolic challenges; Black bears (*Ursus americanus*) and grizzly bears (*Ursus arctos horribilis*) experience massive annual weight gain in preparation for hibernation, grizzly bears can gain in excess of twice their body weight before hibernating, yet they do not develop type-2 diabetes or insulin resistance prior to hibernation.^[5] Male and non-pregnant female polar bears (*Ursus maritimus*) do not hibernate, but polar bears face different challenges, living in the extreme environment of the polar arctic, and have developed strategies to thrive on an enormously lipid rich diet.^[9] Lipids are their predominant energy source and they consume a high fat diet throughout life. Polar bear cubs consume milk containing in excess of 25% fat and adults exist on a diet comprised largely of fatty seals.^[10–11] Adipose deposits can account for up to 50% of the body weight of a polar bear and they cope with lifelong levels of low-density lipoprotein that would be linked to high risk of heart disease in humans.^[9]

We hypothesized that part of the adaptation of polar bears and of bears that live in more temperate climates may involve the neuropancreatic polypeptide hormone amylin (also known as islet amyloid polypeptide or IAPP). Amylin plays an adaptive role in metabolism, but forms islet amyloid in type-2 diabetes in humans, a process which contributes to β -cell dysfunction and loss of β -cell mass in the disease.^[12–17] Loss of insulin production and loss of β -cell mass are key features in the progression of type-2 diabetes, and islet amyloid formation is an important contributor. Islet amyloid formation also contributes to the failure of islet transplants. There are no treatments to preserve β -cell mass and function, or to prevent islet amyloid formation.^[14, 18–21]

Amylin has been found in all mammals studied to date and the sequence is strongly conserved, however not all species form islet amyloid, and those that do not are resistant to type-2 diabetes.^[14] For example, humans, other primates and cats form islet amyloid and develop diabetes, but rodents do not and the difference correlates with variation in the primary sequence of amylin.^[14, 22–23] Rats and mice which are transgenic for human amylin (h-amylin) can be induced to form islet amyloid and become diabetic. Of particular relevance, one mouse model that expresses h-amylin develops extensive islet amyloid and hyperglycemia when fed a high fat diet.^[24] Strikingly the fat level in that study is less than that consumed by polar bears in their normal diet.

The sequence of ursine amylin has recently been reported, providing the opportunity to examine its amyloidogenic properties and its effects on β -cells.^[25] Polar bear and black bear amylin have identical sequences. We show that the ursine hormone is significantly less cytotoxic than h-amylin and is noticeably less amyloidogenic, consistent with the emerging hypothesis that part of the adaptive strategy of bears is to minimize β -cells insults.

2. Results and Discussion

2.1 Ursine Amylin is Significantly Less Amyloidogenic than Human Amylin

Even small changes in the primary sequence of amylin can lead to major changes in the ability of the polypeptide to form amyloid and to induce β -cell death, thus sequence variations which may be deemed minor in globular proteins and other polypeptides can have significant consequences for amylin.^[26] Ursine amylin differs from the human peptide at four positions and contains His-18 Arg, Ser-20 Gly, Phe-23 Leu and Ser-29 Pro substitutions relative to h-amylin (Figure 1, Figure S1). These are interesting changes from a biophysical perspective as the Ser-20 Gly mutation is known to accelerate amyloid formation by human amylin.^[27–29] The Ser-20 Gly mutation is the only known mutation in human amylin and has been linked to a slight increase in risk for diabetes in Japanese populations and has been shown to accelerate amyloid formation *in vitro*. The Ser-20 Gly mutant has recently been shown to be more toxic than h-amylin in a transgenic islet model and in cell culture.^[27, 30] In contrast, the other substitutions are expected to slow amyloid formation by h-amylin. Amyloid formation by h-amylin is slower when His-18 is positively charged and the His to Arg substitution is thus expected to reduce amyloidogenicity since Arg will be fully charged at physiological pH.^[31–34] In addition, the His-18 Arg replacement is one of the substitutions that helps to render rat amylin non-amyloidogenic and this substitution has been exploited to help design soluble non-toxic analogs of amylin.^[22–23, 35] The Ser to Pro

substitution is also expected to reduce amyloidogenicity since Pro disrupts the secondary structure and a Ser-29 Pro mutation is one of the three proline substitution found in the non-amyloidogenic rat amylin (Figure S2).^[22–23] A Phe to Leu replacement is relatively conservative in terms of sidechain size and hydrophobicity. The Phe-23 to Leu mutation has been shown to moderately slow amyloid formation by h-amylin, even though the Phe-23 Leu substitution is found in amylin derived from a number of species that form islet amyloid *in vivo*.^[31–33] The three non His-18 substitutions are all located within the residue 20 to 29 region of the molecule that is believed to be important for modulating amyloidogenicity.^[22–23, 36–37] Thus, it is not clear what the effect of the combined substitutions will be on the amyloidogenicity of ursine amylin. We first analyzed human and ursine amylin using standard amyloid prediction algorithms. The programs either predicted that human amylin is more amyloidogenic than ursine amylin or else predicted that the human polypeptide contains more amyloid prone regions or larger amyloidogenic sequences (Table S1). The programs generally agree that segments within or near residues 13 to 17 and 24 to 27 are parts of amyloidogenic regions. It is clearly important to experimentally test the two polypeptides, however, since the methods fail to predict that certain regions of human amylin are amyloidogenic even though they are part of the core cross- β structure in all existing structures of human amylin amyloid fibrils. In addition, these prediction programs give inconsistent results for other variants of amylin and relative amyloidogenicity can vary depending on the choice of buffer.^[38]

We experimentally examined the ability of the ursine polypeptide to form amyloid using fluorescence detected thioflavin-T binding assays and transmission electron microscopy (TEM). Amyloid formation by amylin and other amyloidogenic proteins is a multi-phasic process composed of a lag phase, in which little or no detectable amyloid fibrils are formed; followed by a growth phase where fibrils lengthen; and a final plateau phase in which amyloid fibrils exist in equilibrium with soluble peptide (Figure S2). Thioflavin-T is a small dye that is widely used in biophysical studies of amyloid formation.^[38–39] The dye is weakly fluorescent in the buffer and does not bind to pre-amyloid intermediates, but does bind to amyloid fibrils and experiences an increase in quantum yield when it does so. Thus, thioflavin-T provides a convenient assay to follow amyloid formation and the dye does not perturb the kinetics of h-amylin amyloid formation under the conditions of our studies. However, thioflavin-T is an extrinsic probe and we, and others, have shown that thioflavin-T assays can lead to false negatives, particularly when studying inhibitors, thus we also used TEM to probe for amyloid formation.^[40–41] TEM directly detects amyloid fibrils and unambiguously confirms their presence or absence.

We conducted initial studies using 20 mM Tris buffer at pH 7.4 at 16 μ M amylin. The pH was chosen to mimic extracellular pH, and because amyloid formation by h-amylin is sensitive to the protonation state of His-18 and the N-terminus.^[31–33] Tris buffer was selected for these studies because it is widely used in biophysical studies of amylin aggregation. h-Amylin forms amyloid with a lag phase on the order of 5.8 ± 0.6 hours, and a T_{50} , the time required to achieve 50 % of the signal change in a thioflavin-T assay, of 12.2 ± 1.9 hours. under these conditions, as judged by thioflavin-T assays, and TEM reveals dense matt of amyloid fibrils (Figure 2). In striking contrast, no increase in thioflavin-T fluorescence intensity was observed for the ursine polypeptide even after one week of

incubation (Figure 2). TEM analysis of aliquots removed after one week confirmed that no amyloid fibrils were present in the ursine amylin sample. We repeated the experiment at significantly higher polypeptide concentration, 100 μ M. Under this condition human amylin had a T_{50} of 4.0 ± 0.2 hours while ursine amylin had a T_{50} of 36.2 ± 2.3 hours (Figure S3). Fewer fibrils were observed on the TEM grid for the ursine sample but regions call be found with contained matts amyloid fibrils appear to be similar at this level of resolution to h-amylin fibrils. The longer T_{50} for u-amylin and h-amylin provides additional evidence that the ursine polypeptide is less amyloidogenic.

The kinetics of amyloid formation by h-amylin are very sensitive to added salt, the choice of salt and, in some cases, the identity of the buffer.^[42] Consequently, we examined amyloid formation by h-amylin and ursine amylin in Tris with 140 mM KCl and in phosphate buffered saline (PBS, 10 mM phosphate 140 mM KCl). As expected, the addition of KCl accelerates amyloid formation by h-amylin and the lag phase of the human polypeptide decreases to 4.5 hours in Tris, 140 mM KCl. No significant increase in thioflavin-T fluorescence is observed for ursine amylin under these conditions even after 1 week of incubation and no fibrils are detected by TEM (Figure S4). In PBS, the lag time for amyloid formation by h-amylin is 4.3 ± 0.1 hours and the T_{50} is 5.2 ± 0.1 hours. No significant increase in thioflavin-T fluorescence is detected for ursine amylin over the week long time course of the experiment. TEM images of a sample of the ursine polypeptide recorded at the end of the kinetic experiment show an absence of large amyloid fibrils, but reveals some small aggregates (Figure 3). In summary, the set of experiments reveals that ursine amylin is considerably less amyloidogenic than the human polypeptide under all conditions tested.

2.2 Anionic Lipid Vesicles Catalyze Amyloid Formation by Human Amylin, But Not by Ursine Amylin

It is well documented that interactions with lipid vesicles that contain a significant mole fraction of anionic lipids catalyze amyloid formation by h-amylin.^[43–45] A reduction in the lag time for amyloid formation and in the T_{50} , the time required to reach 50% of the signal change in a thioflavin-T assay, is observed, particularly for vesicles that contain high, super-physiological mole fractions of anionic lipids. Large unilamellar vesicles (LUV's) containing 25 to 100 mole percent anionic lipid are typically used in such studies. For reference, the fraction of anionic lipids contained in the β -cell plasma membrane has been estimated to be between 2.5 and 13.2 mole percent and they are localized to the inner leaflet.^[46] We compared the ability of mixed anionic zwitterionic LUV's to promote amyloid formation by human and by ursine amylin in order to test if ursine amylin can be induced to form amyloid by changes in environment. A system comprised of 25 mole percent anionic lipid, in this case phosphatidylserine, and 75 mole percent of the zwitterionic lipid phosphatidylcholine was used. We choose this composition because it is well documented to accelerate amyloid formation by human amylin and thus provides a strong amyloid promoting environment. Fluorescence detected thioflavin-T binding assays were used to monitor amyloid formation by h-amylin and ursine amylin in the presence of LUV's. These experiments were carried out using Tris buffer with 100 mM NaCl and a quartz plate. Human amylin forms amyloid with a T_{50} of 43 hours under these conditions in the absence of vesicles. The T_{50} was reduced by a factor of five to 8.3 hours in the presence of LUV's. In

contrast, no detectable increase in thioflavin-T fluorescence was observed over the 90 hour time course of the experiment for the ursine polypeptide (Figure 4). These studies reinforce the results obtained in homogenous solution and provide further evidence that ursine amylin is significantly less amyloidogenic than h-amylin.

2.3 Ursine Amylin is Significantly Less Toxic than Human Amylin

We next compared the effects of ursine and h-amylin on β -cell viability. We used rat INS-1 β -cells, a standard β -cell line widely employed in studies of amylin induced toxicity. Treatment with h-amylin for 24, 48 and 84 hours led to a time-dependent and dose-dependent decrease in β -cell viability as expected (Figure 5, Figure S5). Incubation with 30 μ M h-amylin for 24 hours resulted in significant cytotoxicity, as indicated by the reduction of β -cell viability by 49% compared to control cells. In sharp contrast, 24 hours incubation of the ursine polypeptide proved to be nontoxic, as indicated by a statistically insignificant 7% decrease in β -cell viability (Figure 5). Longer incubation of β -cells with h-amylin led to further reduction in β -cell viability; a 62% decrease was observed for 48 hours and a 70% reduction was measured by 84 hours. The effects of ursine amylin are much less pronounced; incubation with 30 μ M peptide reduced β -cell viability by only 20% after 48 hours of incubation on cells and by 43% after 84 hours of incubation. The effects of both human and ursine amylin were less pronounced at lower peptide concentration, but the human peptide was always significantly more toxic (Figure S5).

We have previously demonstrated that pre-amyloid oligomers of human amylin are more toxic in cell culture than amylin amyloid fibrils. We also showed that not all amylin oligomers are toxic, in particular rat amylin is not cytotoxic, even though rat and human amylin generate similar distributions of low order oligomers.^[47] This led to the hypothesis that properties other than the size of the oligomers are important determinants of toxicity. Ursine amylin provides an additional test of this hypothesis since it is significantly less toxic than the human peptide. We conducted photochemical cross linking studies of ursine and human amylin and observed remarkably similar distributions of oligomeric species (Figure 6), confirming that features beyond just oligomeric sized determine toxicity.

2.4 Mutational Analysis of Amyloid Formation by Ursine Amylin

We analyzed the factors that lead to the reduced amyloidogenicity of ursine amylin relative to h-amylin by preparing a set of point mutants of h-amylin in which each unique residue of ursine amylin was individually substituted into human amylin. Figure 1 compares the sequences of human and ursine amylin and highlights the location of the differences using the high resolution model of the human amylin fibril derived from crystallographic studies of small fragments of human amylin.^[48] The ability of these mutants to form amyloid was tested using thioflavin-T assays and TEM (Figure 7). The effect of the Phe-23 Leu mutation in h-amylin has already been reported as part of a study of the role of aromatic residues in amyloid formation by the human polypeptide.^[37] That mutant has been shown to form amyloid, but at a reduced rate with a T_{50} that is 1.8 times longer than that of h-Amylin. The Ser-20 Gly mutant leads to more rapid amyloid formation, as expected, with no discernible lag phase and with a T_{50} that is only 0.3 that of wild type h-amylin.^[27–29] In contrast, the

His-18 Arg and Ser-29 Pro h-amylin mutants both slow amyloid formation; T_{50} is increased 4.4 fold for His-18 Arg h-amylin and 1.6 fold for Ser-29 Pro h-amylin.

Of the three mutants which slow the rate of amyloid formation, the His-18 Arg substitution has by far the largest effect while the effect of the Phe-23 Leu or Ser-29 Pro substitutions are less, but are still detectable. The plot reveals several interesting features. First, the effect of the Ser-29 Pro mutation is relatively modest despite the fact that a Ser to Pro mutation is very non conservative and despite the fact that Ser-29 is located in the ordered cross β -structure in recent models of the h-amylin amyloid fibril.^[48–50] Second, the effect of the Phe-23 Leu mutation is comparable to, or slightly larger than that of the Ser-29 Pro mutation even though the former is a much more conservative replacement. The sensitivity to substitutions at residue-23 is consistent with recent studies that postulated the transient formation of β -sheet structure in this region during h-amylin amyloid formation and postulated that formation of this structure is a critical step during amyloid formation.^[36]

2.5 Analysis of a His-18 Arg Ser-20 Gly Double Mutant Reveals that the Effect of the His-18 Arg Replacement Dominate the Ser-20 Gly Substitution

We next examined a His-18 Arg Ser-20 Gly double mutant of human amylin since the His-18 Arg and Ser-20 Gly single substitutions had the largest individual effect on the kinetics of amyloid formation by human amylin, with one accelerating aggregation and the other slowing it. A Ser-20 to Gly mutation is the only known mutation in humans and has been reported to lead to a modest increased risk of type-2 diabetes.^[27–28] In contrast, the His-18 to Arg substitution is found in a wide range of non-amyloidogenic amylin (Figure S1). The double mutant behaved much more like the His-18 Arg mutation than the Ser-20 Gly mutation. The value of T_{50} for the double mutant was 54.1 ± 9 hours compared to the values of 46.2 ± 7 hours and 4.5 ± 0.3 hours measured for the His-18 Arg and Ser-20 Gly point mutants respectively. Thus, the His-18 Arg mutant rescues the effect of the Ser to Gly replacement (Figure 8). TEM reveals that the fibrils formed by the double mutant resemble those generated by human amylin and each of the single mutants. The data for all of the mutants is summarized in Figure 9 which displays a bar graph of the T_{50} data for all of the mutants, including data for the Phe-23 Leu h-amylin mutant taken from Tu et al.^[37]

3. Conclusions

The data presented here directly demonstrate that ursine amylin is considerably less amyloidogenic than human amylin and noticeably less toxic to β -cells. These observations are consistent with the hypothesis that reducing the amyloidogenicity and the associated toxicity of amylin may be part of the adaptation of polar bears to cope with an extremely lipid rich diet and part of the adaptation of bears that inhabit more temperate climates to respond to the challenges of hibernation and annual massive weight gain. Of course, the *in vitro* work presented here can not directly prove this conjecture, but it highlights that further experimentation will be of interest. A more rigorous test of this hypothesis would require the generation of rodents transgenic for ursine amylin which lack human amylin. Such a study is beyond the scope of the present work, but it would be particularly interesting to examine the effect of a high fat diet on such an animal. Along these lines, it is mice which are transgenic

for human amylin develop extensive islet amyloid and hyperglycemia when feed a high fat diet, even though that diet has a fat content which is lower than normally consumed by polar bears.^[24]

The effects of the four sequence differences between human and ursine amylin appear to act synergistically in the sense that their effect does not appear to be additive. For a simple two state protein folding reaction deviations from additivity are easily detected by examining the effect of mutations on the rate constant for folding. If the mutations behave independently, they will have additive effects on the log of the rate constant, or equivalently multiplicative effects on the rate constant. Amyloid formation is much more complex than two state protein folding, involving multiple steps, fragmentation, other forms of secondary nucleation and possible parallel pathways with heterogeneous intermediates; thus, it is not possible to directly relate a thioflavin-T curve to a single microscopic rate constant.^[38, 51–52] However, a semi-quantitative comparison can be constructed using the values of T_{50} or the duration of the lag phase.^[37, 53] We analyzed the effect of the single mutations relative to h-amylin by calculating the ratio of the T_{50} of the mutant to the value of T_{50} for human amylin. The expected result if the mutants behave independently is obtained by multiplying the parameters calculated for the four single mutations.^[37, 53] It is important to stress that this analysis is qualitative and assumes that T_{50} can be related to the inverse of a rate constant; none-the-less it does provide insight.^[53] Within the limitations of this approach, independent additive effects are expected to lead to a T_{50} for ursine amylin that will be on the order of 3.8 times longer than observed for h-amylin, as calculated from the experimental data for the four point mutants. However, the T_{50} of ursine amyloid is more than 29 times longer than that observed for h-amylin, strongly suggesting non-additive effects. This calculation is based upon the observed 5.8 hour lag time of h-amylin, together with the fact ursine amylin did not form amyloid for the full week long (168 h) time course of the study. Non-additive effects on solubility have previously been observed for the F23L and S29P mutants in the context of a ten residue peptide fragment consisting of residues 20–29 of h-amylin.^[53]

Analysis of the His-18 Arg and Ser-20 Gly single mutants and the His-18 Arg Ser-20 Gly double mutants also suggests non-additive effects between these two sites. If the effects were additive, then, within the context of this simple analysis, the predicted T_{50} for the double mutant would be between that of His-18 Arg amylin and Ser-20 Gly amylin with a T_{50} on the order of 1.2 longer than human amylin. Instead, the T_{50} of the double mutant is 5.6 times longer than observed for human amylin. The basis for the enhanced rate of amyloid formation by Ser-20 Gly amylin is not understood, thus it is not yet possible to offer a molecular level explanation for the effects of the double mutant. However, residue-18 is located within a region postulated to be important for initial human amylin intermolecular contacts during aggregation.^[54] The insertion of a fully charged residue at this location may lead to charge repulsion that is deleterious for amyloid formation. Poisson-Boltzmann calculations have shown that a protonated His-18 sidechain is very destabilizing to the amyloid fibril while a neutral His sidechain is much more favorable.^[42]

The significant reduction in the amyloidogenicity of the ursine polypeptide and the apparently dominate role of the His-18 Arg replacement have interesting implications for the design of soluble variants of amylin for therapeutic applications.^[35] Amylin is deficient in

type 1 and in type-2 diabetes and is a promising potential adjunct to insulin therapy, but the clinical use of h-amylin is limited by its aggressive tendency to aggregate *in vitro* and because h-amylin aggregates are toxic to β -cells. Less amyloidogenic analogs of h-amylin have been developed by exploiting the sequence differences between rat/mouse and human amylin, but there is still room for improvement. Rat/mouse amylin is not amyloidogenic and is not toxic to β -cells. The rat polypeptide contains three proline substitutions at residues 25, 28, 29 and these are believed to be critical for reducing its amyloidogenicity.^[14, 23]

Pramlintide (also known as Symlin), is a clinically approved variant of human amylin which contains the proline residues found in rat amylin. These substitutions lead to a variant which is non amyloidogenic under typical conditions, administration of pramlintide leads to improve glycemic control in patients.^[55–57] Unfortunately, there are still practical issues with pramlintide. The polypeptide has a limited solubility at neutral pH and is more soluble at acidic pH where His-18 and the N-terminus are fully charged. This presents issues with its formulation, and makes co-formulation with insulin difficult. There is also interest in combining amylin with leptin for the treatment of obesity and co-formulation is difficult in this case because of the poor solubility profiles of human amylin and pramlintide.^[58–60] The biophysical studies of the ursine polypeptide reported here demonstrate that all three proline substitutions are not required to significantly reduce the amyloidogenicity of h-amylin. In addition to the three prolines, rat amylin contains three additional substitutions relative to h-amylin. These include the His-18 to Arg replacement that is found in ursine amylin, as well as a conservative Ile-26 to Val substitution at residue 26 and the same Phe-23 to Leu replacement found in ursine amylin.^[14, 24] Analysis of the point mutants reveals that the Phe-23 to Leu and His-18 to Arg substitution make contributions to reducing the tendency of human amylin to form amyloid, suggesting that these mutations could be exploited in the design of next generation soluble analogs of h-amylin.^[35] Comparison of the Ser-20 Gly and His-18 Arg single mutants to the double mutant highlight the replacement of His-18 as a key design target. The apparent synergy observed between the different substitutions in ursine amylin relative to h-amylin highlights that the effect of multiple mutants on the biophysical properties of h-amylin cannot always be predicted based on the behavior of individual point mutants; an observation which has implications for the design of analogs of h-amylin.

The role of His-18 Arg amyloid formation leads to interesting evolution speculation. A His-18 Arg substitution is found in a range of species (Figure S1). Clearly, a His-18 Arg substitution can be tolerated on functional grounds and clearly it leads to reduced amyloidogenicity, but it is not found in humans. This likely reflects the fact that until relatively recently, type-2 diabetes largely affected people later in life, thereby lessening the evolutionary pressure to reduce factors that contribute to the disease.

Finally, the cross-linking studies confirm that toxicity is dictated by more than just the size of the oligomers which form in the lag phase of amylin amyloid formation.

In conclusion, the reduced cytotoxicity of ursine amylin provides suggestive clues into the potential mechanisms of adaptation of bears to challenging environments and provides insight into strategies for developing soluble, non-toxic analogs of h-amylin. The analysis of other animals which face metabolically challenging environments, but which do not form islet amyloid, should provide additional insight into the origin of the high amyloidogenicity

of the human hormone and will help to further guide efforts towards the rational redesign of human amylin therapeutics.^[21, 61] The reduced toxicity of ursine amylin and the apparently beneficial effects to bears provided additional, albeit indirect, evidence that the prevention of the toxic effects of human islet amyloidosis will have salutary effects in preventing and treating human metabolic disease.

4. Experimental Section

4.1 Peptide Synthesis

Human amylin, ursine amylin, H18R human amylin, S20G human amylin, S29P human amylin and H18RS20G human amylin were synthesized using standard Fmoc (9-fluorenylmethoxy carbonyl) solid phase peptide synthesis methods on a 0.1 mmol scale, with a CEM Liberty automated microwave peptide synthesizer. Fmoc-PAL-PEG-PS resin (0.17 mmol/mg) was used to obtain amidated C-termini. Fmoc protected pseudoproline (Oxazolidine) dipeptide derivatives were used as previously described.^[62] O-(Benzotriazol-1-yl)-N, N, N', N'-tetramethyluronium hexafluorophosphate (HBTU) was used as a coupling agent and 20 % piperidine was used for removal of the Fmoc group. N, N-Dimethylformamide (DMF) was used as the solvent. Standard Fmoc reaction cycles were employed. The first Fmoc protected amino acid attached to the resin, Fmoc protected pseudoproline dipeptide derivatives, and all β -branched Fmoc protected amino acids were double coupled. Peptides were cleaved from the resin and side chains protecting groups were removed using standard trifluoroacetic acid (TFA) methods (91:3:3:3 TFA: Anisole: Thioanisole: 1, 2-Ethanedithiol for 3 h at room temperature). Crude peptides were precipitated with cold diethyl ether and freeze dried. All solvents were ACS grade. Fmoc-Pal-PEG-PS resin was purchased from Applied Biosystems. Fmoc protected amino acids and all other reagents were purchased from AAPPTec, Novabiochem, Sigma-Aldrich, VWR and Fisher Scientific.

4.2 Disulfide Bond Formation and Peptide Purification

Crude peptides were dissolved in 20% acetic acid (v/v) and freeze dried to increase the solubility of the peptide. Crude peptides were then dissolved in 100% dimethyl sulfoxide (DMSO) to promote intramolecular disulfide bond formation and allowed to stand for at least 72 hours at room temperature. The oxidized peptides were purified via reversed-phase HPLC using a C18 preparative column (250 mm $\times$ 20 mm). A two buffer solution system was used. Buffer A was 100% H₂O and 0.045% HCl and buffer B was 80% acetonitrile, 20% H₂O and 0.045% HCl. A gradient of 20% to 60% buffer B in 40 min was used. HCl was used as an ion pairing agent instead of TFA since TFA can influence amylin aggregation kinetics and can also affect amylin cell toxicity studies. To remove residual scavengers, the dried peptides were dissolved in 1, 1, 1, 3, 3, 3-Hexafluoro-2-propanol (HFIP) after the first purification and re-purified with reversed-phase HPLC. The purity of the peptides was checked by reversed-phase HPLC using a C18 analytic column (250 mm $\times$ 4.6 mm). The molecular weights of the purified peptides were confirmed by mass spectrometry using matrix-assisted laser desorption ionization time of flight mass spectrometry (MALDI-TOF MS) instrument: human amylin expected, 3903.30; observed 3902.8; ursine amylin, expected, 3868.3; observed, 3867.2; H18R h-amylin, expected 3922.3; observed, 3921.5;

S20G h-amylin, expected 3873.3; observed 3872.6; S29P h-amylin expected, 3913.3; observed, 3913.0; H18RS20G h-amylin expected 3892.3; observed 3891.6.

4.3 Sample Preparation

Dry peptides (stored at -20°C inside a desiccator) were dissolved in 100 % HFIP to prepare a 1.6 mM stock solution. Aliquots were filtered through a $0.22\text{ }\mu\text{m}$ Millex syringe-driven filter. The concentration of the solutions was determined via by measuring the absorbance at 280 nm. Aliquots were stored at 4°C . Measured amounts of aliquots were freeze dried to remove HFIP. Experiments were initiated by dissolving a dried aliquot of amylin with the appropriate amount of buffer.

4.4 Preparation of Large Unilamellar Vesicles (LUVs)

1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) and 1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-L-serine (POPS) were obtained from Avanti Polar Lipids. Large unilamellar vesicles (LUVs) were prepared from a 75:25 mixtures of POPC and POPS. Lipid stock was dissolved in chloroform in a glass tube at the desired concentration. Stock solutions were evaporated with nitrogen gas and were dried under high vacuum for 2 hours to completely remove the residual organic solvent. The resulting lipid mixtures were then hydrated in Tris buffer (20mM Tris-HCl, 100 mM NaCl at pH 7.4) and agitated at 55°C for 30 minutes to form multilamellar vesicles (MLVs). The MLVs were subjected to 7 freeze-thaw cycles and then extruded through a 100 nm polycarbonate filter (Avanti Polar Lipids) 11 times to obtain uniform vesicle size. The phospholipid concentration was determined by the method of Stewart.^[63] A fresh vesicle solution was used for each experiment.

4.5 Fluorescence Assays

Thioflavin-T binding fluorescence assays were used to follow amyloid fibril formation as a function of time. A Beckman Coulter DTX 880 Multimode Detector plate reader was employed. Thioflavin-T fluorescence was measured using 430 nm excitation wavelength with a filter bandwidth of 35 nm and emission at 485 nm was monitored with a filter bandwidth of 20 nm. Corning 96-well non-binding surface black plates with lids were used for the non-membrane assays. Plates were sealed with polyethylene sealing tape. Freeze dried dry peptides were dissolved in buffer and thioflavin-T solution immediately before the assays. Experiments with 10 mM phosphate buffer and with 140 mM salt were performed in an identical fashion. All the kinetic experiments used: $16\text{ }\mu\text{M}$ amylin, $32\text{ }\mu\text{M}$ thioflavin-T, 25°C , pH 7.4 and no agitation. Uncertainties in the kinetic parameters were estimated from multiple independent measurements.

For membrane assays, samples were incubated in a 96-well black quartz microplate at 25°C . Experiments in the presence of membranes were initiated by adding a solution of $32\text{ }\mu\text{M}$ thioflavin-T, $400\text{ }\mu\text{M}$ LUVs, 20 mM Tris, and 100 mM NaCl, pH 7.4, to dry lyophilized peptide at the final peptide concentration was $20\text{ }\mu\text{M}$. Each experiment was repeated three times using different amylin stock solutions.

4.6 Transmission Electron Microscopy

TEM images were recorded with an FEI Bio TwinG² Transmission Electron Microscope at the Central Microscopy Center at Stony Brook University (Stony Brook, NY). 18 μ L of the peptide solution was taken at the end of the kinetic runs from the samples used for the fluorescence measurements. Samples were blotted on a carbon-coated formvar 300-mesh copper grid for 1 min and then negatively stained with 2% uranyl acetate for 1 min. Images were taken at a 68,000 $\times$ magnification and 100 nm under focus.

4.7 Cytotoxicity Assays

Rat INS-1 β -cells were seeded at a density of 40,000 cells per well in 96-well plates 24 hours prior to the start of experiments. Peptides were dissolved in complete RPMI, and incubated on cells for 24, 48 and 84 hours prior to cell viability measurements. β -cell viability was assessed by AlamarBlue reduction assays. AlamarBlue was diluted ten-fold in culture media and incubated on cells for 5 hours at 37 °C. Fluorescence (excitation 530 nm; emission 590 nm) was measured with a Beckman Coulter DTX880 plate reader. Values were calculated relative to untreated control cells.

4.8 Photo Cross-linking Assays

Cross-linking experiments were performed using Tris(bipyridyl)Ru(II) in conjunction with ammonium persulfate to mediate the photochemical reaction. Lyophilized peptide was dissolved in 20mM Tris HCl (pH 7.4) and centrifuged at 15,000g for 10 minutes prior to cross-linking. The final concentration of peptide in each sample was 32 μ M, with Tris(bipyridyl)Ru(II) and ammonium persulfate in 3.5 $\times$ and 70 $\times$ molar excess respectively. Samples were reacted by exposure to a 150W incandescent bulb for 5 seconds, and subsequently quenched by the addition of β -mercaptoethanol. Cross-linking products were separated by SDS-PAGE using a 10–20% tris-tricine gradient gel, followed by visualization with silver staining (SilverXpress, Invitrogen). Quantitative analysis of oligomer bands was performed using Gelanalyzer software version 2010a. The relative intensities of each band were obtained by integration of the area under each peak, after correcting for the baseline.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

1. Wild S, Roglic G, Green A, Sicree R, King H. Diabetes Care. 2004; 27:1047–1053. [PubMed: 15111519]
2. International Diabetes Foundation. Annual Report. 2013. Available at: <http://www.idf.org/sites/default/files/attachments/IDF-AR2013-final-rv.pdf>

3. Ng M, Fleming T, Robinson M, Thomson B, Graetz N, Margono C, Mullany EC, Biryukov S, Abbafati C, Abera SF, et al. *Lancet*. 2014; 384:766–781. [PubMed: 24880830]
4. Chandrasekera PC, Pippin JJ. *Altex-Altern. Anim. Ex.* 2014; 31:157–176.
5. Nelson OL, Jansen HT, Galbreath E, Morgenstern K, Gehring JL, Rigano KS, Lee J, Gong JH, Shaywitz AJ, Vella CA, Robbins CT, Corbit KC. *Cell Metab*. 2014; 20:376–382. [PubMed: 25100064]
6. Kamine A, Shimozuru M, Shibata H, Tsubota T. *Jpn. J. Vet. Res.* 2012; 60:5–13. [PubMed: 22458193]
7. Martin SL. *Diab. Vasc. Dis. Res.* 2008; 5:76–81. [PubMed: 18537093]
8. Nelson RA. *Mayo Clin. Proc.* 1973; 48:733–737. [PubMed: 4745546]
9. Liu SP, Lorenzen ED, Fumagalli M, Li B, Harris K, Xiong ZJ, Zhou L, Korneliussen TS, Somel M, Babbitt C, Wray G, Li JW, He WM, Wang Z, Fu WJ, Xiang XY, Morgan CC, Doherty A, O'Connell MJ, McInerney JO, Born EW, Dalen L, Dietz R, Orlando L, Sonne C, Zhang GJ, Nielsen R, Willerslev E, Wang J. *Cell*. 2014; 157:785–794. [PubMed: 24813606]
10. Hedberg GE, Derocher AE, Andersen M, Rogers QR, DePeters EJ, Lonnerdal B, Mazzaro L, Chesney RW, Hollis B. *Zoo Biol.* 2011; 30:550–565. [PubMed: 21246608]
11. Thiemann GW, Iverson SJ, Stirling I. *Ecol. Monogr.* 2008; 78:591–613.
12. Westermarck P, Wernstedt C, Wilander E, Hayden DW, O'Brien TD, Johnson KH. *Proc. Natl. Acad. Sci. U. S. A.* 1987; 84:3881–3885. [PubMed: 3035556]
13. Cooper GJS, Willis AC, Clark A, Turner RC, Sim RB, Reid KBM. *Proc. Natl. Acad. Sci. U. S. A.* 1987; 84:8628–8632. [PubMed: 3317417]
14. Westermarck P, Andersson A, Westermarck GT. *Physiol. Rev.* 2011; 91:795–826. [PubMed: 21742788]
15. Cao P, Marek P, Noor H, Patsalo V, Tu LH, Wang H, Abedini A, Raleigh DP. *FEBS Lett.* 2013; 587:1106–1118. [PubMed: 23380070]
16. Akter R, Cao P, Noor H, Ridgway Z, Tu LH, Wang H, Wong AG, Zhang X, Abedini A, Schmidt AM, Raleigh DP. *J. Diabetes Res.* 2016; 2016:2798269. [PubMed: 26649319]
17. Hay DL, Chen S, Lutz TA, Parkes DG, Roth JD. *Pharmacol. Rev.* 2015; 67:564–600. [PubMed: 26071095]
18. Montane J, Klimek-Abercrombie A, Potter KJ, Westwell-Roper C, Verchere CB. *Diabetes Obes. Metab.* 2012; 14:68–77. [PubMed: 22928566]
19. Westermarck GT, Westermarck P, Berne C, Korsgren O, Transpla NNCI. *N. Engl. J. Med.* 2008; 359:977–979. [PubMed: 18753660]
20. Udayasankar J, Kodama K, Hull RL, Zraika S, Aston-Mourney K, Subramanian SL, Tong J, Faulenbach MV, Vidal J, Kahn SE. *Diabetologia.* 2009; 52:145–153. [PubMed: 19002432]
21. Potter KJ, Abedini A, Marek P, Klimek AM, Butterworth S, Driscoll M, Baker R, Nilsson MR, Warnock GL, Oberholzer J, Bertera S, Trucco M, Korbitt GS, Fraser PE, Raleigh DP, Verchere CB. *Proc. Natl. Acad. Sci. U. S. A.* 2010; 107:4305–4310. [PubMed: 20160085]
22. Westermarck P, Engstrom U, Johnson KH, Westermarck GT, Betsholtz C. *Proc. Natl. Acad. Sci. U. S. A.* 1990; 87:5036–5040. [PubMed: 2195544]
23. Betsholtz C, Christmansson L, Engstrom U, Rorsman F, Svensson V, Johnson KH, Westermarck P. *FEBS Lett.* 1989; 251:261–264. [PubMed: 2666169]
24. Verchere CB, DAlessio DA, Palmiter RD, Weir GC, BonnerWeir S, Baskin DG, Kahn SE. *Proc. Natl. Acad. Sci. U. S. A.* 1996; 93:3492–3496. [PubMed: 8622964]
25. Fortin JS, Benoit-Biancamano MO. *Zoo Biol.* 2014; 33:446–451. [PubMed: 25273481]
26. Abedini A, Meng FL, Raleigh DP. *J. Am. Chem. Soc.* 2007; 129:11300–+. [PubMed: 17722920]
27. Sakagashira S, Hiddinga HJ, Tateishi K, Sanke T, Hanabusa T, Nanjo K, Eberhardt NL. *Am. J. Pathol.* 2000; 157:2101–2109. [PubMed: 11106582]
28. Ma Z, Westermarck GT, Sakagashira S, Sanke T, Gustavsson A, Sakamoto H, Engstrom U, Nanjo K, Westermarck P. *Amyloid.* 2001; 8:242–249. [PubMed: 11791616]
29. Cao P, Tu LH, Abedini A, Levsh O, Akter R, Patsalo V, Schmidt AM, Raleigh DP. *J. Mol. Biol.* 2012; 421:282–295. [PubMed: 22206987]

30. Meier DT, Hogan MF, Templin AT, Kahn SE. Diabetes. 2015; 64:A576–A576.
31. Charge SBP, Dekoning EJP, Clark A. Biochemistry. 1995; 34:14588–14593. [PubMed: 7578065]
32. Abedini A, Raleigh DP. Biochemistry. 2005; 44:16284–16291. [PubMed: 16331989]
33. Jha S, Snell JM, Sheftic SR, Patil SM, Daniels SB, Kolling FW, Alexandrescu AT. Biochemistry. 2014; 53:300–310. [PubMed: 24377660]
34. Green J, Goldsbury C, Mini T, Sunderji S, Frey P, Kistler J, Cooper G, Aebi U. J. Mol. Biol. 2003; 326:1147–1156. [PubMed: 12589759]
35. Wang H, Abedini A, Ruzsicska B, Raleigh DP. Biochemistry. 2014; 53:5876–5884. [PubMed: 25140605]
36. Buchanan LE, Dunkelberger EB, Tran HQ, Cheng PN, Chiu CC, Cao P, Raleigh DP, de Pablo JJ, Nowick JS, Zanni MT. Proc. Natl. Acad. Sci. U. S. A. 2013; 110:19285–19290. [PubMed: 24218609]
37. Tu LH, Raleigh DP. Biochemistry. 2013; 52:333–342. [PubMed: 23256729]
38. Wong AG, Wu C, Hannaberry E, Watson MD, Shea JE, Raleigh DP. Biochemistry. 2016; 55:510–518. [PubMed: 26694855]
39. LeVine H. Methods Enzymol. 1999; 309:274–284. [PubMed: 10507030]
40. Meng F, Marek P, Potter KJ, Verchere CB, Raleigh DP. Biochemistry. 2008; 47:6016–6024. [PubMed: 18457428]
41. Hudson SA, Ecroyd H, Kee TW, Carver JA. FEBS J. 2009; 276:5960–5972. [PubMed: 19754881]
42. Marek PJ, Patsalo V, Green DF, Raleigh DP. Biochemistry. 2012; 51:8478–8490. [PubMed: 23016872]
43. Hebda JA, Miranker AD. Annu Rev Biophys. 2009; 38:125–152. [PubMed: 19416063]
44. Jayasinghe SA, Langen R. Biochemistry. 2005; 44:12113–12119. [PubMed: 16142909]
45. Brender JR, Dertouzos J, Palfey B, Ballou DP, Massey V, Entsch B, Gafni A, Steel DG. Biophys. J. 2005; 88:167a–167a.
46. Seeliger J, Weise K, Opitz N, Winter R. J. Mol. Biol. 2012; 421:348–363. [PubMed: 22321797]
47. Abedini A, Plesner A, Cao P, Ridgway Z, Zhang J, Tu LH, Middleton CT, Chao B, Sartori DJ, Meng F, Wang H, Wong AG, Zanni MT, Verchere CB, Raleigh DP, Schmidt AM. Elife. 2016; 5
48. Wiltzius JJW, Sievers SA, Sawaya MR, Cascio D, Popov D, Riekel C, Eisenberg D. Protein Sci. 2008; 17:1467–1474. [PubMed: 18556473]
49. Luca S, Yau WM, Leapman R, Tycko R. Biochemistry. 2007; 46:13505–13522. [PubMed: 17979302]
50. Alexandrescu AT. PLoS One. 2013; 8:e56467. [PubMed: 23457571]
51. Arosio P, Vendruscolo M, Dobson CM, Knowles TP. Trends Pharmacol. Sci. 2014; 35:127–135. [PubMed: 24560688]
52. Michaels TC, Lazell HW, Arosio P, Knowles TP. J. Chem. Phys. 2015; 143:054901. [PubMed: 26254664]
53. Ashburn TT, Lansbury PT. J. Am. Chem. Soc. 1993; 115:11012–11013.
54. Wiltzius JJW, Sievers SA, Sawaya MR, Eisenberg D. Protein Sci. 2009; 18:1521–1530. [PubMed: 19475663]
55. Weyer C, Maggs DG, Young AA, Kolterman OG. Curr. Pharm. Des. 2001; 7:1967–1967.
56. Fineman MS, Koda JE, Shen LZ, Strobel SA, Maggs DG, Weyer C, Kolterman OG. Metabolism. 2002; 51:636–641. [PubMed: 11979398]
57. Schmitz O, Brock B, Schmitz O. Diabetes. 2004; 53:S233–S238. [PubMed: 15561917]
58. Seth R, Terry DE, Parrish B, Bhatt R, Overton JM. Brain Res. 2012; 1442:15–24. [PubMed: 22306030]
59. Trevaskis JL, Lei CL, Koda JE, Weyer C, Parkes DG, Roth JD. Obesity. 2010; 18:21–26. [PubMed: 19543217]
60. Roth JD, Roland BL, Cole RL, Trevaskis JL, Weyer C, Koda JE, Anderson CM, Parkes DG, Baron AD. Proc. Natl. Acad. Sci. U. S. A. 2008; 105:7257–7262. [PubMed: 18458326]
61. Gerstein HC, Waltman L. Can. Med. Assoc. J. 2006; 174:25–26. [PubMed: 16389231]

62. Marek P, Woys AM, Sutton K, Zanni MT, Raleigh DP. *Org. Lett.* 2010; 12:4848–4851. [PubMed: 20931985]
63. Stewart JC. *Anal. Biochem.* 1980; 104:10–14. [PubMed: 6892980]

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Figure 1.

(a) Comparison of the primary sequence of human and ursine amylin. Both peptides have an amidated C-terminus and an intramolecular disulfide bond between Cys-2 and Cys-7. (b) A top down view of a model of the amylin amyloid fibril based on crystal structures of small fragments of amylin.^[47] His-18, Gly-20, Phe-23, and Ser-29 are shown in space filling format together with some of the key residues they interact with. (c) A side view, in ribbon format, of the structure. The N-terminal β -strand in each monomer is colored coded red, the C-terminal β -strand green, while the disordered N-terminal region and the loop which connects the two β -stands are color coded blue.

Figure 2.

Ursine amylin is less amyloidogenic than human amylin and does not form amyloid in Tris buffer. (a) Thioflavin-T fluorescence experiments comparing the kinetics of amyloid formation by human amylin (●) and ursine amylin (●). TEM images recorded at the end of the experiments for (b) human amylin and (c) ursine amylin. Scale bar = 100 nm. Experiments were conducted at pH 7.4, 25 °C and 20 mM Tris.

Figure 3.

Ursine amylin is much less amyloidogenic than human amylin in phosphate buffered saline. (a) Thioflavin-T fluorescence experiments comparing the kinetics of amyloid formation by human amylin (●) and ursine amylin (●). TEM images recorded after 7 days of the experiments for (b) human and (c) ursine amylin. Scale bars are 100 nm. Experiments were performed in 10 mM phosphate, 140 mM KCl, pH 7.4 and 25°C.

Figure 4.

Anionic lipid vesicles catalyze amyloid formation by human amylin, but not by ursine amylin. Thioflavin-T assays comparing the kinetics of amyloid formation by human amylin (●) and ursine amylin (●) in the presence (open circles) and absence (solid circles) of vesicles. The data for ursine amylin in the presence and absence of LUVs overlaps. LUVs contained 75 mol % POPC, 25 mol % POPS and the total lipid concentration was 400 μ M. Experiments were conducted in 20 mM Tris, 100 mM NaCl, pH 7.4 at 25 °C with 20 μ M peptide.

Figure 5.

Ursine amylin is less toxic to β -cells than human amylin. Human amylin (■) and ursine amylin (■) were incubated on rat INS-1 β -cells for 24, 48, 84 hours (left to right) and cell viability was assessed using AlamarBlue assays. Final peptide concentration on cells was 30 μ M. Data is normalized to untreated control cells (■).

Figure 6.

Ursine amylin shows similar oligomeric distributions in solution as human amylin though ursine amylin is less amyloidogenic and less toxic than human amylin. (a) Ursine amylin and human amylin formed low order oligomeric species separated by SDS-PAGE gels: lane-1, markers (molecular weight: kDa); lane-2, human amylin and lane-3, ursine amylin. (b) Analysis of oligomeric species distribution in gels: human amylin (●) and ursine amylin (●). Experiments were conducted in 20 mM Tris buffer at pH 7.4 with 32 μ M peptide. Data represents mean for 9 measurements of h-amylin and 2 measurement of u-amylin.

Figure 7.

Mutational analysis of amyloid formation by ursine amylin. (a) Thioflavin-T fluorescence experiments comparing the kinetics of amyloid formation by human amylin (●); ursine amylin (●); H18R human amylin (●); S20G human amylin (●); S29P human amylin (●). TEM images of (b) H18R human amylin, (c) S20G human amylin, and (d) S29P human amylin. A TEM image of wild type human amylin is found in figure 2b. Scale bar = 100 nm. Experiments were conducted at pH 7.4, 25 °C and 20 mM Tris.

Figure 8.

Comparison of the rate of amyloid formation by human amylin (●), H18R human amylin (●), S20G human amylin (●) and the H18RS20G double mutant (●). (a) Thioflavin-T kinetics experiment. (b) TEM image of the H18RS20G double mutant. TEM images of the single mutants are found in Figure 7. Scale bar = 100 nm.

Figure 9.

Bar graph comparing the value of T_{50} for wild type human amylin, H18R human amylin, S20G human amylin, F23L human amylin, S29P human amylin and H18RS20G human amylin. The data is plotted as $T_{50}/(T_{50}$ of human amylin) and was collected in 20 mM Tris, pH 7.4 and 25°C. The data for the F23L mutant is from reference.^[37] Data represents mean and error bars represent the standard deviation, for 3 measurements.