Growth-incompetent monomers of human calcitonin lead to a noncanonical direct relationship between peptide concentration and aggregation lag time

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Kian Kamgar-Parsi, Liu Hong, Akira Naito, Charles L. Brooks III, and Ayyalusamy Ramamoorthy

From the 4 Applied Physics Program, University of Michigan, Ann Arbor, Michigan 48109, 6 Zhou Pei-Yuan Center for Applied Mathematics, Tsinghua University, Beijing 100084, China, 6 Graduate School of Engineering, Yokohama National University, 79-5 Tokiwadai, Hodogaya-ku, Yokohama 240-8501, Japan, and 1 Department of Chemistry and Biophysics Program, University of Michigan, Ann Arbor, Michigan 48109-1055

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The role of the peptide hormone calcitonin in skeletal protection has led to its use as a therapeutic for osteoporosis. However, calcitonin aggregation into amyloid fibrils limits its therapeutic efficacy, necessitating a modification of calcitonin’s aggregation kinetics. Here, we report a direct relationship between human calcitonin (hCT) concentration and aggregation lag time. This kinetic trend was contrary to the conventional understanding of amyloid aggregation and persisted over a range of aggregation conditions, as confirmed by thioflavin-T kinetics assays, CD spectroscopy, and transmission EM. Dynamic light scattering, 1H NMR experiments, and seeded thioflavin-T assay results indicated that differences in initial peptide species contribute to this trend more than variations in the primary nucleus formation rate. On the basis of kinetics modeling results, we propose a mechanism whereby a structural conversion of hCT monomers is needed before incorporation into the fibril. Our kinetic mechanism recapitulates the experimentally observed relationship between peptide concentration and lag time and represents a novel mechanism in amyloid aggregation. Interestingly, hCT at low pH and salmon calcitonin (sCT) exhibited the canonical inverse relationship between concentration and lag time. Comparative studies of hCT and sCT with molecular dynamics simulations and CD indicated an increased α-helical structure in sCT and low-pH hCT monomers compared with neutral-pH hCT, suggesting that α-helical monomers represent a growth-competent species, whereas unstructured random coil monomers represent a growth-incompetent species. Our finding that initial monomer concentration is positively correlated with lag time in hCT aggregation could help inform future efforts for improving therapeutic applications of CT.

Calcitonin (CT) is a 32-amino acid peptide hormone produced in thyroidal c-cells (1). Originally discovered due to its hypocalcemic effects, further studies showed CT’s main function to be skeleton-protective (1). CT inhibits bone resorption and maintains bone mass through G protein-coupled receptor-mediated interactions with osteoclasts, and it has therefore been used as a therapeutic for the bone diseases osteoporosis and Paget’s disease (1–4). However, CT-based therapies have been limited by the intrinsic tendency of CT to aggregate in solution as both the structural reformatting and sequestration of CT during its aggregation decrease bioavailability and efficacy (5, 6).

The characteristics of CT’s aggregation mark it as a member of the amyloid family. These peptides and proteins are characterized by their propensity to form amyloid fibrils, in which monomeric subunits adopt β-strand structures and stack along the fibril axis through intermolecular hydrogen-bonding and electrostatic interactions (7). The structure of these fibrils and the general process by which they form from their respective monomeric subunits is thought to be relatively conserved across different primary sequences (7, 8). Briefly, during the initial phase of aggregation known as the lag phase, peptide monomers self-associate to form small, soluble aggregates (oligomers), with continued aggregation leading to the production of protofibrils. Such protofibrils then seed rapid elongation through monomer addition, leading to the formation of mature amyloid fibrils. Crucially, the fibrillation of amyloidogenic peptides has been shown to play a role in a number of disease pathologies. At least 60 such peptides have been identified in humans, with more than half having been implicated in diseases including Alzheimer’s, Parkinson’s, and type 2 diabetes mellitus (9–13). Despite extensive investigations, significant confusion remains as to the details of aggregation and the specific roles that such peptide aggregates play in disease pathology. The heterogeneous and metastable nature of the prefibrillar aggregates as well as the large effects that subtle shifts in environmental factors have on aggregation have combined to obfuscate the identification of critical aggregation intermediates or specific

The abbreviations used are: CT, calcitonin; hCT, human calcitonin; sCT, salmon calcitonin; hIAPP, human islet amyloid polypeptide; CMC, critical micelle concentration; ThT, thioflavin-T; TEM, transmission electron microscopy; DLS, dynamic light scattering; EGCG, epigallocatechin gallate.

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toxic species. A detailed elucidation of amyloid aggregation would both aid in disease understanding and provide guidance in efforts to inhibit aggregation and toxicity.

For CT in particular there is a need to slow aggregation to improve therapeutic efficacy. To this end the rapidly aggregating human calcitonin (hCT) has been replaced by the slower aggregating salmon calcitonin (sCT) as the current standard in calcitonin-based osteoporosis therapy (14, 2). Despite improvements over hCT, sCT-based therapies are associated with side effects and immune responses that interfere with therapeutic efficacy (4, 15, 16). Additionally, hCT maintains improved therapeutic efficacy compared with sCT when aggregation is controlled and is free of the side effects and immune responses associated with sCT (5, 17). Significant interest therefore exists in slowing the aggregation of hCT to improve its utility as a therapeutic agent.

Here we show for the first time a direct relationship between peptide concentration and lag time in the aggregation of hCT. Such a trend goes against the conventional wisdom of the amyloid field, where increasing concentrations lead to faster aggregation (18, 19, 20, 21). In several other amyloids, including the closely related human islet amyloid polypeptide (hIAPP), “micelle-like” oligomers formed above a critical micelle concentration (CMC) have emerged as potential key kinetic-modulating intermediates; however, such intermediates in hCT were found to be kinetically inactive (19, 20, 22, 23). Instead, our results point to the need for monomer structural conversion as responsible for the trend. We investigated the concentration-dependent and structural differences in these species and propose a novel kinetic model for amyloid formation by hCT. Further elucidation of this aggregation pathway could identify key intermediates and inform future efforts in kinetics modification in the therapeutic applications of CT.

**Results**

**hCT presents an atypical direct relationship between initial monomer concentration and lag time**

Thioflavin-T (ThT) assays have become the standard for kinetic monitoring of amyloid fibril growth due to the quantum yield increase in ThT upon binding to β-sheet rich structures (24–28). We used ThT assays to probe the concentration dependence of hCT aggregation rates. ThT kinetic curves were measured for a range of initial monomer concentrations (Fig. 1A). The ThT traces of hCT show the typical sigmoidal fibril growth pattern characteristic of amyloids; however, the direct relationship between peptide concentration and lag time (time duration of the lag phase) is contrary to the expected behavior (Fig. 1B). It is well-established that the aggregation characteristics of amyloids depend heavily on their aggregation environment, yet the atypical trend observed for hCT is found to persist through changes of buffer (concentration and type), salt concentration, pH, agitation, and temperature (supplementary Fig. S1) (29, 30). This consistency indicates the behavior to be characteristic of hCT rather than a consequence of a specific set of environmental conditions.
To ensure that the ThT data were reflective of the macroscopic peptide behavior rather than a report on a limited sub-population, population-wide secondary structure was determined via CD spectroscopy. The time evolution of secondary structure was measured for 30 μM and 120 μM initial monomer concentrations (Fig. 1, C–D). At both concentrations, hCT initially displayed a primarily random coil secondary structure, typical for monomers of CT (31, 32, 33). Aggregation was accompanied by a depletion in the random coil form and a subsequent increase in β-sheet signal for both peptide concentrations, confirming the ThT-detected increase in β-sheet to be representative of the overall peptide population. The β-sheet signal was stable for at least 24 h, indicating the hCT to have reached its mature fibrillar state. Consistent with the trend found from ThT fluorescence data, the 120 μM hCT took approximately 4 times longer to undergo the transition to a β-sheet conformation than the 30 μM hCT. For both conditions, significant signal loss occurred due to the aggregation and gelation of the peptide, a phenomenon previously described for CT (34). To ensure signal loss did not introduce artifacts into secondary structure determinations, end stage β-sheet morphology was verified by single-point ThT fluorescence (data not shown).

TEM images were also taken to further confirm the validity of our ThT and CD results. Aggregation of 30 μM and 120 μM hCT was tracked by ThT fluorescence, and samples were removed for imaging at equivalent stages of aggregation for both concentrations (supplemental Fig. S2). Early and late lag phase images show short, ThT-negative, protofibrillar aggregates of a few hundred nanometers in length (Fig. 1, E–H). The protofibrils are morphologically indistinguishable between early and late lag phase for both concentrations, with the exception of concentration-dependent density differences. Final stage aggregates show long, thin amyloid aggregates forming fibrils (Fig. 1, I–J). Again, the aggregate morphology remains the same in both samples. Both concentrations exhibited the canonical progression of fibril formation via nucleating protofibrils and elongation, with no discernable concentration-dependent differences in morphology at any stage. Combined with the ThT assays, our CD and TEM results confirm the direct relationship between peptide concentration and lag time to be a genuine behavior.

**Anomalous lag time dependence on peptide concentration is not entirely primary nucleus-dependent**

The observed hCT concentration-dependent differences in lag time combined with the conserved elongation rates are consistent with theoretical predictions of differences in primary nucleation rate (35). The lack of concentration-dependent morphological differences in protofibrillar or fibrillar aggregates by TEM or CD supports the idea that concentration-dependent changes in lag time involve the rate of nucleus formation rather than the characteristics of the nucleus itself (36). To probe this hypothesis, seeded ThT kinetics assays were performed (37, 38, 39). Although the addition of seeds accelerated fibril formation, an extended lag phase remained even in the presence of high seed concentrations (supplemental Fig. S3A). Additionally, in the presence of seed the monomer concentration dependence of the lag phase was abolished (supplemental Fig. S3B). A lag time independent of free monomer concentration is expected for seeded ThT assays and is consistent with differences in primary nucleation rate contributing to the direct relationship between lag time and peptide concentration. However, the persistence of an extended lag time even with 15% seeding by volume implies that the existence of primary nuclei is not sufficient for fibril formation and elongation, with such a phenomenon having been previously shown in other systems (40).

**hCT oligomer behavior changes with concentration**

The results of our seeded ThT kinetics assays focused further analysis on the presence and interconversion of oligomeric intermediates that dominate the lag phase. To probe the kinetics of oligomerization rather than fibril formation, the time evolution of hCT aggregate size was monitored using Dynamic light scattering (DLS) experiments. Despite peptide preparations minimizing the number of non-monomeric species initially present, peaks representing small oligomeric species and larger aggregates appear rapidly after sample preparation at both high (120 μM) and low (30 μM) hCT concentrations (Fig. 2A). To confirm the detected species as hCT aggregates rather than artifacts, epigallocatechin gallate (EGCG), a natural product known to interact with and break apart amyloid aggregates including oligomers of hCT, was added (31, 41, 42). The addition of EGCG shifted the small oligomeric peak from 2 to 5 nm to <1 nm in radius, confirming the source of the peak to be small soluble aggregates (Fig. 2A). The peak associated with larger, potentially protofibrillar aggregates was unaffected by EGCG. Previous studies have revealed the effect of EGCG on hCT aggregates to be mediated primarily by interactions between the aromatic rings of EGCG with those of Tyr-12 and Phe-16 (31). The ability of EGCG to remodel only low molecular weight aggregates by DLS would suggest the larger aggregates to be significantly more stable, potentially due to the sequestration and subsequent solvent inaccessibility of the aromatic residues.

In EGCG-free preparations, two clear differences in aggregate size appear. First, the smaller peak representing low molecular weight oligomeric species shows consistently larger aggregates for 120 μM hCT (4–5-nm radius) as opposed to 30 μM (1–2 nm radius) (Fig. 2B). Such a finding suggests that the oligomeric population is concentration-dependent, favoring larger oligomers at higher concentrations. Second, the observed changes in the larger peak initially appearing at ~100 nm for both conditions indicate a significantly faster growth for samples containing 120 μM hCT compared with those containing 30 μM, which suggests a more rapid reorganization of aggregates to larger species at higher concentrations (Fig. 2C). DLS results do not exhibit any statistically significant concentration- or time-dependent differences in mass distribution between peaks. The majority of peptide mass (~80%) is contained in peak I as expected for early aggregation stages (supplemental Fig. S4), indicating that the relative percentage of peptide in each peak remains fixed over the time-course measured in the experiment.
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(A) Mass % vs. Radius (nm)

(B) Particle Size (nm) vs. Time (min) for 120 µM hCT and 30 µM hCT

(C) Particle Size (nm) vs. Time (min) for 30 µM hCT and 120 µM hCT

(D) ¹H Chemical Shift (ppm) over time:
- t = 0 min
- t = 40 min
- t = 80 min
- t = 120 min
- t = 160 min
- t = 210 min
- t = 420 min
- t = 600 min

(E) ¹H Chemical Shift (ppm) for different time points:
- t = 0 hr
- t = 3 hr
- t = 6 hr
- t = 9 hr
- t = 12 hr
- t = 15 hr
- t = 18 hr
- t = 21 hr

(F) Intensity vs. Time (min):
- t = 0 hr
- t = 21 hr

(G) ¹H Chemical Shift (ppm) with time:
- t = 0 hr
- t = 21 hr
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To further probe the concentration-dependent time evolution of hCT oligomers, a time-course 1D $^1$H NMR experiment was used to track the progression of oligomerization by monitoring the peak at $-0.1$ ppm. This peak appears in the NMR spectra of amyloidogenic peptides when aliphatic protons are shielded from solvent, as occurs when they are sequestered in oligomeric aggregates (31, 28, 43). Monitoring the time evolution of this peak at high and low concentrations provides information on the concentration dependence of oligomerization kinetics specifically, an alternative to typical kinetic assays that track only fibrillation kinetics.

At both concentrations an oligomer peak was found immediately, consistent with the DLS data. Over time, the oligomer peak intensity decreased for both peptide concentrations (Fig. 2, D and F). Interestingly, the $^1$H NMR spectra revealed a slower loss of oligomer peak intensity at higher concentrations (Fig. 2F). This would initially appear as a trivial result given that the ThT and CD time-course experiments both exhibit slower aggregation at higher concentrations, implying a longer period of oligomer-dominated peptide distribution and slower loss of signal intensity to NMR-invisible species. However, the overall spectrum signal intensity for both concentrations is unchanged over the course of the experiment (Fig. 2G), indicating the differential rates in oligomer peak intensity loss to be independent of fibrillation and instead representative of concentration-dependent differences in oligomeric reformatting. The authors note that given the NMR-invisible size of the larger, rapidly growing aggregate species detected by DLS (peak II), the changes in the oligomer peak detected by NMR must represent a different process involving changes in either oligomer packing or prevalence. Taken together, both the DLS and the NMR data confirm concentration-dependent differences in early oligomeric behavior independent of primary nucleus formation. Specifically, higher hCT concentrations exhibit larger and longer lived oligomers during the lag phase than lower hCT concentrations.

The effect of micelle-like oligomers on hCT aggregation

Given a postulated oligomeric culprit driving the abnormal hCT aggregation, we investigated a potential role for micelle-like oligomers. In contrast to amyloid oligomers as typically described, which form over a range of concentrations with varied sizes and morphologies, micelle-like oligomers form exclusively above their CMC. Additionally, such oligomers consistently result in the shielding of hydrophobic peptide regions in the oligomeric interior, analogously to lipid micelles. Studies have reported that several amyloid proteins including amyloid-$\beta$, hAPP, and PrP form such oligomers, with these oligomers exhibiting significant effects on aggregation kinetics (19, 20, 22, 23). The formation of micelle-like oligomers has also been proposed, but not detected, for hCT and provides a potential mechanism for the concentration versus lag time trend (30, 44). At higher concentrations an elevated number of micelle-like oligomers could pull progressively larger amounts of monomers off-pathway, providing a sink for monomers and resulting in slower aggregation.

We sought to determine whether or not micelle-like hCT oligomers form by monitoring the fluorescence spectrum of pyrene, a well-established technique for detecting micelle formation (19, 45, 46). Pyrene emission spectra were collected with changing peptide concentrations, and the peak I/peak III intensity ratio was plotted against concentration (Fig. 3). This ratio underwent a sharp decrease upon crossing $\sim 80 \mu M$ peptide, indicating that micelle-like hCT oligomers form above this CMC. The pyrene spectral shift occurs at roughly the same concentration with both increasing (titration) and decreasing (dilution) peptide concentrations, indicating that the formation of the micelle-like oligomer is reversible. This formation of a reversible micelle-like oligomer of hCT is consistent with the behavior of similar aggregates in other amyloids and represents
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the first direct confirmation of micelle formation by hCT (19, 20, 22).

Despite confirming their formation, our results to this point suggest that micelle-like oligomers of hCT play no role in the anomalous concentration-dependent aggregation trend observed. The relationship between lag time and peptide concentration displays no discontinuity at or above the CMC as would be expected if micelle-like oligomers were involved (Fig. 1B). This kinetic inactivity of the micelle-like oligomer of hCT is in odds with the behavior of similar aggregates in other amyloids and casts doubt as to its role in previously proposed hCT aggregation pathways.

Inhibition by growth-incompetent hCT monomers explains the anomalous trend in concentration versus lag time

The micelle independence of the atypical concentration versus lag time trend combined with the persistence of a long lag phase in the presence of seeds necessitates another determinant of lag time. The existence of no apparent concentration-dependent morphological differences in protofibrillar or mature fibril species suggests the canonical elongation and fragmentation mechanisms for amyloid fibril kinetics to be at work (35). Additionally, the persistence of a prominent lag time in the presence of seeds would suggest that initial hCT peptides are different from classical monomers in that they cannot immediately be incorporated into fibrils during elongation. Such a difference could arise through the need for a structural conversion step in the monomers before they are growth-competent. As such, we propose that the inverse dependence of lag time on the monomer concentration is explained by the existence of two distinct types of monomers, growth-competent and growth-incompetent (see “Experimental procedures” for model details). Such promiscuity in monomer conformation inhibits the formation of mature fibrils by both limiting the availability of growth-competent monomers and through the formation of slowly reversible growth-incompetent aggregates (Fig. 4A). Such a model accurately reproduces our experimental data showing a direct relationship between lag time and monomer concentration (Fig. 4, A and B). Details of model species evolution, rate constant influence, and sensitivity analysis are provided in supplemental Figs. S5–S7.

Comparisons between our model and other unsuccessful models are shown in Fig. 4, C and D, highlighting the necessity of key model elements. We also note that the removal of the monomer conversion step in our model leads to a reversion to the canonical relationship between peptide concentration and lag time, as has been previously shown in the literature (35, 47).

Molecular dynamics (MD) simulations implicate the α-helical structure as critical for monomer growth competence

To correlate our study with other kinetics studies focused on maintaining monomeric CT for therapeutic applications, we compared the behavior of hCT with the therapeutically preferred sCT (Fig. 5A). As expected, sCT exhibited significantly longer lag times than hCT under identical conditions (Fig. 5B). However, despite the significant sequence homology, under identical conditions sCT displays the canonical relation between lag time and peptide concentration for amyloids, in contrast to hCT. According to our kinetics modeling, the reversion of sCT to the canonical relationship between peptide concentration and lag phase should be correlated with a difference in the initial structures in sCT as compared with hCT. Experimental methods to distinguish specific monomer or oligomer conformations in the interconverting and heterogeneous pre-fibrillar milieu have yet to be achieved in amyloid research. To circumvent these limitations, MD simulations were utilized to probe the relative distributions of hCT and sCT monomers in solution. MD simulations show sCT to sample a more compact region of conformational space and occupy fewer structural basins (clusters) on its energy landscape than hCT, with 82% of sCT conformations falling in the 4 most populated clusters compared with just 26.4% for hCT (Fig. 5, D and E, supplemental Fig. S8A). sCT monomer conformations had smaller radii of gyration and a more compact distribution of end-to-end distances than hCT (supplemental Fig. S8, C and D). sCT also shows several discrete overrepresented end-to-end distance values relative to a normal Gaussian distribution, further hinting at increased structure. Significantly, sCT monomers show increased numbers of helix-incorporated residues as compared with hCT, a finding in keeping with previous comparative studies (supplemental Fig. S8B) (48, 49). This increased helical structure in sCT versus hCT immediately after peptide solubilization was confirmed by CD measurements (Fig. 6). The decreased conformational heterogeneity and more structured helical sCT monomer as compared with hCT is consistent with the kinetic model predictions of differing degrees of monomer heterogeneity, leading to the canonical inverse relationship between lag time and concentration in sCT. Specifically, the increased helical structure in the sCT monomer would suggest that this more structured “sCT-like” monomer represents the growth-competent species, whereas the less ordered “hCT-like” species represents the growth-incompetent species, which requires conversion through oligomeric intermediates to gain structure and proceed to fibril incorporation.

Although the precise causes for these differences between hCT and sCT remain unknown, one possible explanation lies in the mutation of the three central aromatic residues in hCT (Tyr-12, Phe-16, Phe-19) to Leu in sCT (50, 51). The ability for EGCG to dissociate early aggregates in hCT, believed to be associated with monomer conversion, suggests that aromatic residues play a key role in said conversion as well. As such, we performed MD simulations on a hCT mutant (Y12L, F16L, F19L) to probe the role of these residues in the monomer conformational heterogeneity and structure. Interestingly, the mutated hCT behaved in a fashion intermediate to hCT and sCT; the mutant displayed decreased conformational heterogeneity, increased α-helical content, and decreased radii of gyration and end-to-end distances as compared with wild-type hCT, rendering it more sCT-like (Fig. 5F, supplemental Fig. S8). Our MD simulations thus support the idea that these residues are important for not only interpeptide-stabilizing interactions but also as intrapeptide conformational constraints.
Figure 4. Monomer conversion step allowed accurate kinetic modeling of observed behavior. A, reaction diagram for hCT fibrillation and comparison between experimental (colored squares) and computational (dashed lines) evolutions show model to recapitulate experimental trend. Global fitting (dashed lines) was performed according to Equation 1 with $k_{n} = 1.8 \times 10^{-3}$ h$^{-1}$, $k_{e} = 1 \times 10^{5}$ M$^{-1}$ h$^{-1}$, $k_{mc} = 3.5 \times 10^{5}$ h$^{-1}$, $k_{b} = 1.2 \times 10^{5}$ M$^{-1}$ h$^{-1}$, $k_{b} = 1 \times 10^{-1}$ h$^{-1}$, $k_{f} = 1 \times 10^{-1}$ h$^{-1}$, and $n_{s} = 1$. B, comparison of dependence of lag time on peptide concentration between experimental and computational results. Lag time for both computational and experimental data is determined according to fitting with Equation 1. C, removal of lateral structure conversion from protofibril to mature fibril will cause a gradual decrease in predicted curve slopes. The best global fitting is shown according to Equation 1 with $k_{n} = 4.9 \times 10^{-3}$ h$^{-1}$, $k_{e} = 1 \times 10^{2}$ M$^{-1}$ h$^{-1}$, $k_{mc} = 58.3$ h$^{-1}$, $k_{b} = 1.3$ h$^{-1}$, $k_{f} = 1.6 \times 10^{-2}$ h$^{-1}$, and $n_{s} = 2.3$. D, removal of the monomer inhibition step on protofibril elongation will cause the disappearance of the inverse relationship between lag time and peptide concentration. Curves are drawn based on best global fitting with $k_{n} = 4.9 \times 10^{-3}$ h$^{-1}$, $k_{e} = 1 \times 10^{2}$ M$^{-1}$ h$^{-1}$, $k_{mc} = 58.3$ h$^{-1}$, $k_{b} = 1.3$ h$^{-1}$, $k_{f} = 1.6 \times 10^{-2}$ h$^{-1}$, and $n_{s} = 2.3$. 

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(A) hCT: CGNLSTCMG Gly-TYNDFNKFH TPNTAIGVG

sCT: CNSLSTCVL Gly-KLSQELHKLQ TYPRNTGSG

(B) Normalized Fluorescence vs Time (min)

(C) Normalized Fluorescence vs Time (min)

(D) (E) (F) (G)
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Our studies instead suggested that although helix formation is key in hCT aggregation, structural differences in free monomers, rather than an aggregate-mediated sequestration, results in the increase in lag time with higher peptide concentration.

The existence of growth-competent and growth-incompetent monomers has been proposed previously; however, several novel aspects of our model warrant further consideration (55). The reproduction of our reported trend requires the monomer conversion rate to depend inversely on initial peptide concentration, with a relatively slow rate constant. Such a phenomenon is unsurprising if we assume monomer conversion to occur indirectly through the formation and dissociation of low molecular weight oligomeric intermediates. According to the Ostwald ripening mechanism, the dissociation of larger particles is slower than that of smaller ones, with the time being roughly proportional to particle size (56, 57). As such, we would expect the rate of oligomeric reformating, and thus monomer conversion, to be inversely proportional to oligomer size. Not only is this differential rate of oligomer reformating seen in our time-course NMR experiments, but the larger size of initial small oligomers observed at higher concentrations by DLS provides direct evidence for the existence of such an intermediate species. The inverse dependence of the kinetic term $k_{m_{tot}}$ on $m_{tot}$ incorporates these results into our kinetic model.

Additionally, our model proposes the existence of off-pathway protofibrillar species, which persist without immediately elongating to fibrils. In particular, we propose these off-pathway species to be formed by the addition of growth-incompetent monomers to growth-competent aggregates, essentially providing a means by which such monomers inhibit further growth and elongation. The detection of larger ThT-negative aggregates in the lag phase by DLS and TEM such as those predicted by the model during the lag phase, before mature fibril formation and elongation, is consistent with this model. Interestingly, the lack of a ThT-detectable β-sheet signal from these protofibrils as well as the marked morphological differences between protofibrils and mature fibrils hints at possible structural conversion steps in addition to the monomer conversion. Such a lateral structure conversion is fully consistent with the requirement of a unimolecular reaction, which accounts for the concentration independence of elongation rate observed in our ThT traces.

Interestingly, our MD simulations show both the hCT mutant and hCT at low pH to form an amphipathic α-helix in the central region between Asp-15 and Phe-19, whereas such a helix is absent in the wild-type hCT. This central DFNKF region (DLNKL in the hCT mutant) forms the core of hCT fibrils and has previously been shown to adopt a helical conformation in monomers as determined by NMR (44, 58, 59). Our results suggest that the helical structure of the DFNKF region may be key in determining the growth competence of hCT monomers.
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Figure 7. ThT kinetics assays at pH 5.4 indicated the direct relationship to remain. Assays were performed in triplicate using citric acid buffer (pH 5.4) at 25 °C, with orbital shaking and 2 eq of ThT. The similar relationship between lag time and concentration at pH 7.4 and 5.4 indicates the behavior to be independent of the charge state of His-20 (pKa = 6).

Calcitonin exhibits non-canonical aggregation behavior potentially by driving initial monomer self-recognition events through hydrophobic interactions. Low pH environments and leucine residues have both previously been shown to enhance helical propensity, consistent with our findings (60, 61). In particular for hCT going from pH 7.4 to pH 3.3, the side chains of Asp-15 and His-20, both, become protonated. Given the proximity of these residues to the central helix forming region, they present likely causes for the pH-dependent change. ThT kinetics experiments at pH 5.4 (where His-20 is protonated but Asp-15 is not) reveal the maintenance of the direct relationship between concentration and lag time, suggesting that the charge state of Asp-15 plays the larger role in the pH-determined growth competence of hCT monomers(Fig. 7). Further experiments involving the above-mentioned hCT mutant and other, more structured hCT monomers will be valuable in fully understanding the role of monomer structure and concentration in determining lag time.

Previous studies have reported both hCT and sCT to exhibit α-helical structural motifs during the progression to mature β-sheet conformations, consistent with our predictions (6, 62–67). We note that the dominance of random coil structure in our CD experiments with smaller amounts of helical structure are not contradictory to these studies. The predicted monomer structures of hCT and sCT, both here and in other studies, maintain extended unstructured regions, with limited numbers of helical residues. Additionally, the relatively rapid incorporation of converted monomers into fibrils, a mechanism consistent with previous amyloid research and exemplified in supplemental Fig. S5A, would serve to preferentially sequester helical monomers in favor of less structured monomers.

In light of the fact that low pH conditions and mutated hCT have been shown to slow the aggregation of hCT, it is important to note that we do not propose the shift to a more sCT-like growth-competent monomer to represent an overall acceleration of the aggregation pathway but, rather, a loss of need for monomer reformattting and thus a return to the canonical relation between peptide concentration and lag time. Despite this, a correlation does exist between the degree of monomer structure and lag time. We speculate that the more stable helices in sCT-like monomers, although encouraging initial monomer interactions and aggregation events, could resist conversion to β-sheet structures, thus delaying overall lag times. This explanation finds support in the reported delay of sCT aggregation by helical dimers, mediated by the helical propensity of the monomer and stabilizing hydrophobic leucine interactions between helices (54, 68). It seems likely that our observations of monomer helical stability being correlated with slower lag times are due to the formation of similar stable helical structures, but further studies are required to confirm the persistence of this behavior in our experiments.

The reported relationship between concentration and lag time in hCT also holds therapeutic promise through the possibility for higher effective doses of hCT without the correlated hindrance of decreased bioavailability. Indeed such a phenomenon has broad interest for all fibrillating therapeutic peptides, e.g. insulin, where large local peptide concentrations and subsequent aggregation pre-uptake, for example within nanoparticles, pose obstacles to therapeutic efficacy and alternative therapeutic delivery methods (69–73). With the increasing prevalence of therapeutic peptides in the pharmaceutical market, such issues are taking on progressively more importance. The direct relationship we see between lag time and peptide concentration could inform future efforts to design appropriate peptide delivery systems and allow the packaging of higher dosages of peptide without loss of efficacy.

Finally, it appears that micelle-like oligomers play no significant role in hCT aggregation, in contrast to other amyloids. In addition to being kinetically inactive, our CD, NMR, and TEM experiments showed no structural or morphological differences upon the introduction of micelle-like oligomers through increased concentration. TEM measurements do show punctate proteinaceous aggregates in the late lag phase above the CMC only, with such aggregates being absent at low concentrations (supplemental Fig. S9). Such aggregates may be the micelle-like oligomers but show no spatial association with other aggregates, further suggesting a non-functional role. Naito and co-workers (30) previously observed spherical aggregates of hCT to form in HEPES buffer. Such oligomers were shown to seed fibril formation, and micelle-like oligomers were proposed as a preceding species to these spherical aggregates. Given the lack of kinetic influence or fibril association of our micelle-like oligomers, it would seem that the spherical aggregates formed in HEPES buffer actually form in a micelle-independent fashion. The kinetic inactivity of micelle-like oligomers of hCT observed in this study is especially confounding given recent work highlighting the role of such oligomers in the aggregation of the related peptide hIAPP (20). Both hCT and hIAPP belong to the calcitonin family, bind to the same receptor, contain an N-terminal disulfide bond, and possess similar numbers of residues and charges. The causes for this deviation in oligomer influence are unknown and are confounded by the reversible formation of oligomers and the difficulty in isolating them from the pre-fibrillar milieu.

In summary, we have for the first time demonstrated a direct relationship between initial monomer concentration and lag time for hCT. ThT kinetic assays revealed this trend to exist in
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A range of aggregation environments, and the differential rates in fibril formation were confirmed by CD and TEM measurements. The micelle-like oligomers detected in hCT do not influence its aggregation kinetics, unlike the strong effects reported for similar oligomers in other amyloids. Reseeded ThT assays along with DLS and NMR results suggested an origin for the aggregation behavior in the early steps of aggregation. Kinetic modeling provided a mechanism whereby monomer conformation conversion and subsequent inhibition slows aggregation in a concentration-dependent process. hCT at low pH and sCT were both found to show the canonical inverse relationship between peptide concentration and lag time. Our results suggest that small structural elements in the mostly unstructured monomers of CT could be necessary for fibril formation. Future efforts to ascertain a more detailed structural understanding of this atypical behavior of hCT and overcome the difficulties inherent in detecting such monomeric reformatting in the heterogeneous amyloid environment are needed to fully characterize the aggregation process.

Experimental procedures

Peptide preparation

Human calcitonin peptide was synthesized via Fmoc chemistry as described previously (31). The disulfide bond between Cys-1 and Cys-7 of hCT was formed by air oxidation at pH 8.0–8.5 at diluted concentrations and in the presence of 6 M urea to prevent fibrillation. To ensure consistent monomeric starting conditions, peptide was dissolved in hexafluoroisopropanol (HFIP) at a concentration of 1 mg/ml. Peptide was then separated into 100-μl aliquots, flash-frozen in liquid nitrogen, and lyophilized for a period of 48 h. After lyophilization, peptide aliquots were stored at −20 °C until use. Peptide solutions were prepared by resolubilizing the lyophilized aliquots in 100 μl of HCl (pH 4) at 4 °C to a concentration of ~300 μM followed by brief sonication to ensure peptide dissolution. Peptide solutions were then warmed to room temperature and subsequently used.

Thioflavin-T kinetic assay

ThT experiments were run on a Biotek Synergy 2 microplate reader (BioTek Instruments, Inc., Winooski, VT) on uncoated Fisherbrand 96-well polystyrene plates. Peptide was added to final concentrations of 20 mM buffer and ThT at 25 °C and immediately before acquisition. Experiments were performed in duplicate with 2 eq of ThT and 100-μl total volume unless otherwise indicated. Wells were bottom-read with an excitation wavelength of 440 nm (30 nm bandwidth) and emission wavelength of 485 nm (20 nm bandwidth). Curves were normalized and averaged, and kinetic parameters tlag and tso were calculated via fitting to the equations (24).

$$p(t) = p_\infty + \frac{p_0 - p_\infty}{1 + e^{k(t - t_{lag})}}$$  \hspace{1cm} (Eq. 1)

For seeded assays, seeds were generationally created to ensure uniform morphology (74). First generation seeds were created at 120 μM in 20 mM phosphate buffer at a 50-μl volume at 37 °C shaking at 1000 rpm for 24 h. Subsequent generations were seeded by the previous generation at 10% by volume and incubated under identical conditions. Fourth generation seeds were seeded identically with a 200-μl final volume. Fourth generation seeds were used for ThT kinetics assays.

Circular dichroism

CD spectra were taken on a Jasco J-1500 Circular Dichroism Spectrometer (JASCO, Inc., Easton, MD). Spectra were recorded at 25 °C with 120 μM hCT and 30 μM hCT concentrations in 20 mM phosphate buffer (pH 7.4) in a 1-mm quartz cuvette unless otherwise indicated. Spectra were recorded with a 2-nm bandwidth, 1-nm step size, scan speed of 100 nm/min, baseline-corrected, and averaged over 5 scans. Smoothing was performed using the Savitzky-Golay method, with a convolution width of 15. Between reads, peptide samples were transferred to a microcentrifuge tube and vortexed for 2 s every 10 min.

Transmission electron microscopy

Samples for TEM were removed at three separate time points from a single well of a ThT plate and flash-frozen in liquid nitrogen. Glow-discharged grids (Formvar/carbon 300-mesh, Electron Microscopy Sciences, Hatfield, PA) were treated with the samples for 2 min at room temperature. Excess sample was removed with filter paper and washed three times with double-distilled H2O. Each grid was stained with uranyl acetate (1% w/v, double-distilled H2O, 5 min, 1 min), blotted to remove excess stain, and dried for 15 min at room temperature. TEM images were recorded on a JEOl 1400-plus TEM (80kV) (Microscopy and Image Analysis Laboratory, University of Michigan, Ann Arbor, MI).

Dynamic light scattering

DLS measurements were taken at 25 °C using a DynaPro NanoStar Dynamic Light Scattering Spectrometer (Wyatt Technology, Santa Barbara, CA). All reagents and buffers were syringe-filtered (0.22-μm pore size) before sample preparation. Initially monomeric aliquots of hCT were added to a final peptide concentration of 120 μM or 30 μM in 20 mM phosphate buffer (pH 7.4) and immediately loaded into the cuvette for analysis. Scattering was measured every 2 min for the first hour, every 6 min for the following 2 h, and every 10 min for the subsequent 2 h. All reported measurements are averages of 10 acquisitions. Trials with indistinguishable oligomer and larger aggregate peaks or with poor fits were disregarded in analysis.

Nuclear magnetic resonance

All NMR spectra were obtained on a Bruker 600-MHz spectrometer (Bruker Corp., Billerica, MA). 300 μl of peptide solution in 20 mM phosphate buffer (pH 7.4) with 10% D2O (v/v) was prepared. Spectra were collected every 2 min at 25 °C with...
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A spectral width of 8992.8 Hz and 64 or 32 scans for the 30 μM and 120 μM samples, respectively. Spectra were processed using Topspin 2.1 (Bruker), where normalized oligomer peak intensities were determined via fixed bound integration.

Pyrene fluorescence assay

Pyrene solutions were prepared by dissolving pyrene powder in DMSO followed by serial dilution down to 1 μM in 20 mM phosphate buffer (pH 7.4). Samples were prepared by dilution of peptide stock to a final peptide concentration in 20 mM phosphate buffer (pH 7.4) with 1 μM pyrene to ensure constant pyrene concentration throughout titration/dilution. Pyrene emission spectra were recorded with a FluoroMax-4 Spectrofluorometer (Horiba, Ltd., Kyoto, Japan) with final spectra obtained via the averaging of 3 scans taken at 25 °C. Excitation was at 334 nm with a bandwidth of 2 nm; emission bandwidth was 1 nm. The intensities of peaks I and III were recorded at 370 nm. The intensities of peaks I and III were recorded at 370 nm and 381 nm, respectively. The I/III intensity ratio was plotted versus peptide concentration, and the CMC was determined by fitting the data to an inverse logarithmic decay and determining the point at which the ratio dropped beyond low concentration average.

Kinetics modeling

The kinetics modeling equations are given below.

\[ m_\text{t}(t) = -k_{\text{m}} m_\text{t}(0) + k_\text{m} m_\text{t}(t)P_\text{f}(t) + k_0^{-1} P_\text{f}(t) \]  
\[ m_\text{s}(t) = k_{\text{m}} m_\text{t}(0) - n_k m_\text{s}(t)\text{MC} - 2k_\text{m} m_\text{t}(t)P_\text{f}(t) \]  
\[ P_\text{f}(t) = k_\text{m} m_\text{s}(t)\text{MC} + k_b m_\text{s}(t) - k_\text{m} m_\text{t}(t)P_\text{f}(t) + k_\text{b} P_\text{f}(t) \]  
\[ \dot{P}_\text{b}(t) = k_\text{b} m_\text{s}(t)\text{MC} + k_\text{b} m_\text{s}(t) - k_\text{b} P_\text{f}(t) \]  
\[ \dot{M}_\text{fr}(t) = n_k m_\text{s}(t)\text{MC} + 2k_\text{m} m_\text{t}(t)P_\text{f}(t) - k_\text{n} M_\text{fr}(t) \]  
\[ \dot{M}_\text{fr}(t) = k_c M_\text{fr}(t) \]

In these equations, \( m_\text{t}(t) \) and \( m_\text{s}(t) \) represent the concentrations of hCT peptides before and after structure conversion, \( P_\text{f}(t) \) and \( P_\text{b}(t) \) represent the number concentration of growth-incompetent and growth-competent aggregates, and \( M_\text{fr}(t) \) and \( M_\text{fr}(t) \) represent the mass concentrations of protofibrils and mature fibrils, respectively. \( m_\text{tot} \) represents the total concentration of hCT peptides in the system. It is noticeable that \( m_\text{tot} = m_\text{t}(t) + m_\text{s}(t) + M_\text{fr}(t) + M_\text{fr}(t) \) due to the conservation law of mass. \( k_{\text{m}}, k_\text{m}, k_\text{b}, k_{\text{b}}, k_\text{n}, k_\text{n} \), and \( k_{\text{c}} \) are the rate constants governing the structure conversion of monomers and protofibrils, the primary nucleation of hCT monomers, the conversion from growth-competent to growth-incompetent aggregates by binding with pre-converted hCT monomers and its inverse reaction the growth of fibrils through elongation, and the fragmentation of protofibrils, respectively (Fig. 4A). \( n_\text{c} \) represents the critical nucleus size for primary nucleation.

Molecular dynamics simulations

MD simulations were performed using the CHARMM software package and force fields (75, 76). To sample the conformational distributions adopted by each peptide in solution, simulations were performed utilizing the replica exchange methodology and an implicit, generalized Born solvent model adopted to run on GPUs (77, 78). The peptide was represented in an implicit (Debye-Huckel) ionic environment representing 100 mM NaCl (79). All heavy atom–hydrogen bonds were constrained using the SHAKE algorithm, and the each temperature window of the replica exchange sampling was coupled to a Langevin temperature bath at the desired temperature (80, 81). Twelve temperature windows, which were exponentially distributed between 310 K and 400 K, were employed. Each replica was sampled for 100 ns or a total sampling of 1.2 μs. For each replica 5000 snapshots were saved at each temperature for subsequent analysis. The conformational distribution for each sequence was characterized at 310 K by computing the distribution of structural characteristics, i.e. end-to-end distance, helical content, and radius of gyration. Additionally, the ensemble of structures at 310 K from each sequence was visualized by clustering the structures from replica-exchange steps 1000–5000 using K-means clustering with 4.5 Å Cα + Cβ root mean square based with the MMTSB Tool cluster.pl criterion (82).

Author contributions—K. K.-P. and A. R. conceived the idea and planned the study. K. K.-P. performed the ThT, CD, NMR, DLS, and TEM experiments. L. H. carried out kinetics modeling, and C. L. B. III performed the MD simulations. K. K.-P. and A. R. analyzed the experimental data and interpreted the results. K. K.-P., L. H., C. L. B. III, and A. R. wrote the paper. A. N. provided the peptides. A. R. directed the project. All authors reviewed the results and approved the final manuscript.

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