Converting the Highly Amyloidogenic Human Calcitonin into a Powerful Fibril Inhibitor by Three-dimensional Structure Homology with a Non-amyloidogenic Analogue*

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Irreversible aggregation limits bioavailability and therapeutic activity of protein-based drugs. Here we show that an aggregation-resistant mutant can be engineered by structural homology with a non-amyloidogenic analogue and that the aggregation-resistant variant may act as an inhibitor. This strategy has successfully been applied to the amyloidogenic human calcitonin (hCT). Including only five residues from the non-amyloidogenic salmon calcitonin (sCT), we obtained a variant, polar human calcitonin (phCT), whose solution structure was shown by CD, NMR, and calculations to be practically identical to that of sCT. phCT was also observed to be a potent amyloidogenesis inhibitor of hCT when mixed with it in a 1:1 ratio. Fibrillation studies of phCT and the phCT-hCT mixture mimicked the sCT behavior in the kinetics and shapes of the fibrils with a dramatic reduction with respect to hCT. Finally, the effect of phCT alone and of the mixture on the intracellular cAMP level in T47D cells confirmed for the mutant and the mixture their calcitonin-like activity, exhibiting stimulation effects identical to those of sCT, the current therapeutic form. The strategy followed appears to be suitable to develop new forms of hCT with a striking reduction of aggregation and improved activity. Finally, the inhibitory properties of the aggregation-resistant analogue, if confirmed for other amyloidogenic peptides, may favor a new strategy for controlling fibril formation in a variety of human diseases.

The intrinsic propensity of peptides and proteins to irreversibly aggregate limits the development of protein-based drugs because aggregation compromises their bioavailability and therapeutic activity and increases the risk of immunogenic reactions (1, 2). Possible strategies for overcoming these problems involve the design of specific analogues in which the physicochemical properties of the molecule are changed through mutations of a small number of amino acids (3) or the development of safe inhibitors such as small peptide fragments (4–6). However, because at the moment large molar excesses of inhibitors are used, their pharmacological efficacy appears to be of limited relevance.

A good example of a bioactive peptide with limited pharmaceutical potential due to a high tendency to aggregate is human calcitonin (hCT). It is a 32-residue hormone synthesized and secreted by the C cells of the thyroid and involved in calcium regulation and bone dynamics (7). In its common form it presents an N-terminal disulfide bridge between positions 1 and 7 and a C-terminal proline amide residue. Only eight residues are common to all species so far studied, and these are clustered at the two ends of the molecule. The salmon variant (sCT) is widely used in the treatment of osteoporosis and Paget disease as well as malignancy-caused hypercalcemia and musculoskeletal pain (8, 9). This is because hCT shows an extremely high tendency to form amyloid fibrils both in vivo in patients with medullar carcinoma of the thyroid (10) and in vitro in preparations designed for patient administration (11). Therefore, aggregation constitutes a serious problem that leads to a significant decrease in the hormone activity (12). Moreover, aggregation stimulates undesirable immune responses, resulting in resistance or allergic reactions in patients (13, 14), and drug-induced cytotoxicity (15). On the contrary, sCT shows a higher potency combined with a longer in vivo half-life when compared with hCT and a substantially lower propensity to aggregate (7, 11), but it has been shown to develop side effects such as anorexia and vomiting (16, 17). When aggregation is prevented by drastic chemical conditions, hCT shows a much higher potency than sCT (18); however, those conditions are difficult to implement during the production, storage, and administration to patients, explaining why hCT has never been extensively used as a therapeutic.

Here we demonstrate that it is possible to convert hCT into an aggregation-resistant mutant by including in hCT the

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structural determinants of the non-amyloidogenic sCT with the mutant itself becoming an inhibitor of hCT amyloid formation. Both hCT and sCT take up an amphipathic $\alpha$-helix of different lengths in the central region and an extended C-terminal tail. However, although the hCT helix is flexible, in sCT, it is stable and interacts with the tail (19). Such an arrangement in sCT could be considered a “self-protection” (20) that prevents the C terminus from remaining in an extended conformation to become a template for fibril formation (11, 21, 22). The hCT mutant was engineered by changing five hCT key residues, identified with Waltz software (23), at sites 12, 17, 26, 27, and 31 with the corresponding sCT residues, and the mutant was called polar hCT (24) and referred to as pHCT (Fig. 1). With these mutations we were able to induce in pHCT a solution structure presenting a helix-tail interaction as observed in sCT. More importantly, pHCT mimicked the sCT behavior in the kinetics and shapes of the fibrils and was shown to be a potent inhibitor of hCT amyloidogenesis when mixed with wild-type hCT in a 1:1 ratio, and both pHCT and the pHCT-hCT mixture showed a dramatic reduction of amyloid tendency with respect to hCT. Finally, the effect of pHCT and the pHCT-hCT mixture on the intracellular cAMP level in T47D cells confirmed for the pHCT and the mixture their calcitonin-like activity, exhibiting a stimulation effect identical to that of sCT, the major therapeutic form. The strategy of developing aggregation-resistant analogues of hCT by structure homology with the non-fibrillating sCT is of interest as they keep a high sequence identity with the native hCT. Furthermore, the possibility of using a mixture of aggregation-resistant mutant and wild-type hCT in therapy might avoid undesirable immune responses induced by fish calcitonins and/or the use of drastic formulation conditions and high concentration of antifibrillating agents. Finally, the inhibitory properties of the aggregation-resistant analogue with structure homology with a non-fibrillating analogue, if confirmed for other amyloidogenic peptides, open new possibilities for controlling fibril formation in a variety of human diseases.

EXPERIMENTAL PROCEDURES

Peptide Synthesis—pHCT, hCT, and sCT were synthesized at INBIOS S.r.l. (Naples, Italy). LasCT, in which Pro$^{23}$ and Arg$^{24}$ were substituted for Leu and Ala, respectively, belonged to a batch described previously (20). Peptide purity was confirmed by reversed phase HPLC and MALDI-TOF MS using a Bruker Biflex instrument (Bremen, Germany) in the linear mode at 19.5 kV.

Thioflavin T (ThT) Fluorescence—Calcitonins were dissolved in 1,1,3,3,3, hexafluoropropanol to yield a 420 $\mu$M stock solution and incubated at room temperature for 10 min. After sonication for 1 min in a bath sonicator to ensure disociation of any preaggregated structures, stock solutions were distributed into working solutions with a final concentration of 10 $\mu$M in 10 mM Tris buffer. Aliquots of working solutions were diluted 10-fold into a Tris buffer with 0.75 $\mu$M ThT. Fluorescence values were measured using a Jobin Yvon Horiba Fluoromax 3 fluorometer immediately after preparation and after various time periods using an excitation of 450 nm and an emission of 480 nm. Each experiment was performed with four independent repeats. Average values are presented with bars indicating the S.D.

Circular Dichroism (CD) Spectroscopy—Measurements were performed on an AVIV 202 spectropolarimeter (Aviv Instruments, Lakewood, NJ) connected to a water bath used to control the temperature of the cell. Spectra were recorded in the far UV region (200–250 nm) at 310 K and pH 7.4 (10 mM Tris, 100 mM NaCl) with a peptide concentration of 10 $\mu$M in a 10-mm-path length cell. Measurements were carried out immediately after preparation and after various time periods. A blank spectrum of the buffer was subtracted from each sample spectrum. A spectral bandwidth of 1.0 nm and a scan speed of 10 nm/min were used. Five scans were averaged, and the results are reported as mean residue ellipticity ($\theta$). Prediction of percentages of secondary structure from CD spectra was obtained using the k2d software, a Kohonen neural network with a two-dimensional output layer (25).

Transmission Electron Microscopy—A 10-µl aliquot of each sample, taken at variable times after the reaction had commenced and at the final time point, was placed on a 400-mesh copper grid covered by carbon-stabilized Formvar film (SPI Supplies, West Chester, PA). After 2 min of incubation, excess fluid was removed, and the grid was negatively stained with 10 ml of 2% (w/v) uranyl acetate for 2 min, washed once with 10 ml of Milli-Q water, blotted, and allowed to dry in air. Grids were viewed using a JEOL 1200EX electron microscope operating at 80 kV and a high resolution Philips Tecnai F20 field emission gun transmission electron microscope operating at 200 kV.

Cell Culture and Cyclic AMP Assay—Human ductal breast cancer T47D cells were cultured as described (26) in RPMI 1640 medium containing 10% heat-inactivated FBS, 1% streptomycin/penicillin, 0.1 $\mu$M insulin, and 0.1 $\mu$M hydrocortisone in 5% CO$_2$ at 310 K. The latter hormones were omitted from the medium when subculturing cells to be used 1–3 days later for the cAMP assay. Subculturing was performed with trypsin/EDTA as described (27) in multiwell plates until cells reached 90% confluence (1–3 days after subculture), and then cells were washed in Dulbecco’s PBS-glucose (Gibco, Invitrogen) to remove possible trace of FBS.

Sample solutions were prepared by dissolving different amounts of peptides in Dulbecco’s PBS-glucose supplemented with 0.1% BSA and 1 mM 3-isobutyl-1-methylxanthine. After being washed, cells were incubated for 15 min at 310 K in the presence of peptide solutions. After aspiration of the medium, cAMP was extracted from the monolayers with 500 µl of ice-cold 95% ethanol, 1 mM HCl for 1 h at 283 K. Extracts were then concentrated in a SpeedVac and reconstituted, and cAMP was determined by radioimmunnoassay as described (28).

The reversibility of the interaction of peptides with CT receptors was analyzed by using a three-phase incubation procedure (3). First, cells were incubated for 15 min at 310 K in 200 ml of binding medium with 100 nM peptides or left untreated. Then they were washed three times with 500 ml of binding medium and incubated for 6 h at 310 K with two washing steps every 2 h, resulting in a total of nine washing
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steps. Cells were then incubated for 15 min at 310 K in 200 ml of binding medium containing 1 mM 3-isobutyl-1-methylxanthine with or without 100 mM unlabeled peptide. cAMP was then determined as described above. This procedure resulted in three groups of treatments: (i) cells stimulated without pretreatment (acute), (ii) unstimulated cells after pretreatment (recovered), and (iii) cells stimulated after pretreatment (restimulated).

Waltz Analysis—To predict amyloidogenic regions in hCT mutants, the primary sequences, in the FASTA format, were directly submitted to the Waltz (23). Specific amyloidogenic sites of the hCT amino acidic sequence were obtained by statistical analysis of the aggregation properties as derived from the Waltz software. The sequences obtained by non-contiguous single or multiple substitutions in the wild-type hCT were evaluated with Waltz to predict the amyloidogenicity of the mutants and identify the site(s) that are expected to prevent aggregation. We first evaluated the effects of mutations for the complete hCT sequence, generating ~6000 analogues. The times that a site/region was found to prevent fibrillation according to Waltz (i.e. when a substitution abolished fibrillation) were plotted against the hCT sequence as “occurrence (%),” therefore identifying the most probable aggregation-resistant sites/regions. To better define them, we limited the mutations to the Met8–Phe19 and the Ala26–Ala31 regions found to contain the most probable (>50%) sites in the analysis of the above ~6000 analogues. This reduced set consisted of ~2000 sequences. From the non-fibrillating analogues predicted by Waltz, we derived an occurrence >80% for residues located at sites 12, 17, 26, 27, and 31, which were then selected to convert hCT into phCT.

NMR Experiments—For acquisition of NMR spectra, the concentration of phCT in 95% 1H2O, 5% 2H2O (CortecNet) and 100% 2H2O was 1.5 × 10−3 M. Solid perdeuterated SDS (Cambridge Isotope Laboratories, Woburn, MA) was added, and its concentration was maintained well above the critical micelle concentration with a peptide-SDS molar ratio of about 1:100.

1H NMR spectra were recorded at 600 MHz on a Bruker DRX-600 spectrometer equipped with a TCI CryoProbeTM fitted with a gradient along the Z-axis. Spectra were referenced to internal sodium 3-(trimethylsilyl)-[2,2,3,3-2H4]propionate (Aldrich). Clean total correlation spectroscopy (TOCSY) (29) and NOESY (30) spectra were recorded by using the time-proportional phase incrementation of the first pulse and incorporating the excitation sculpting sequence (31) for water suppression. We used a double pulsed field gradient echo with the gradient pulses of 1 ms each field gradient echo with a soft square pulse of 4 ms at the water resonance frequency with the gradient pulses of 1 ms each in duration. In general, 512 equally spaced evolution time p tendrá; values were acquired, averaging eight transients of 2048 points with 6024 Hz of spectral width. Time-domain data matrices were all zero-filled to 4096 points in both dimensions, thus yielding a digital resolution of 2.94 Hz/point. Prior to Fourier transformation, a Lorentz-Gauss window with different parameters was applied for both t1 and t2 dimensions for all the experiments. NOESY spectra were obtained with different mixing times (0.10, 0.20, and 0.25 s);

TOCSY experiments were recorded with spin-lock periods of 64 and 96 ms achieved with the MLEV-17 pulse sequence. Both NOESY and TOCSY experiments were performed at 300, 310, and 324 K to remove cross-peak overlap. The coordinates of phCT energy-minimized conformers have been deposited in the Protein Data Bank (code 2JXZ).

Structure Calculations—Experimental NOE intensities in phCT were manually assigned and converted into proton-proton distance restraints, which were classified into six ranges, 1.8–2.6, 1.8–3.0, 0.18–0.36, 0.18–0.43, 0.18–0.48, and 0.18–0.53 nm, corresponding, respectively, to strong, strong-medium, medium, medium-weak, weak, and very weak NOEs. Intensities were calibrated using as reference distance of 0.36 nm the αGln14-NHAsp15 cross-peak expected in the middle of a regular α-helix. This hypothesis was confirmed by interproton distance analysis on all resulting models and on experimental 1JHNOx coupling constants. The distance ranges were derived using as intensity cutoff peak integral values corresponding to 9x, 3x, 1x, x/3, x/6, and x/9 the reference value, respectively. The shortest range was increased from 0.25 to 0.26 nm after analysis of several short range distances in the helical region of the preliminary models. A similar analysis suggested using 0.29 nm as the upper limit value for vicinal intraresidue αCH2-HN, medium intensity peaks. The initial 219-NOE data set was iteratively increased after the assignment of ambiguous NOE cross-peaks, leading to a total of 244 experimentally derived distance restraints. The methyl and methylene protons as well as all proton sets with degenerate chemical shifts (e.g. αCH and βCH of Thr23) were described using an “ambiguous restraint” approach in which for each restraint all possible distances between the two methylene (or three methyl) protons and their NOE counterpart proton(s) were calculated and weighted by the inverse of the sixth power of their values. Subsequently, the structural analysis of the best conformers with the program MOLMOL (32) allowed the stereospecific assignment of nine of 16 nondegenerate methylene proton pairs, namely the β-methylene pairs of residues 4, 7, 8, 9, 12, 19, and 20; the α protons of Gly10; and the γ-methylene protons of Met8, by correlating distances obtained from the calculated structures with NOE intensities. As we only refined the major all-trans conformer (see “Results”), all peptide bonds were forced into a trans conformation (ω = 180°) by torsional constraints with a force constant of 200 kJ mol−1 operating for deviations higher than 20° from the trans form. Chirality restraints were also applied to ensure proper amino acids chiralities and prochiralities.

Structures were sampled and refined with a restrained simulated annealing protocol using the Sander_classic module of AMBER 6 (33) using the AMBER all-atom-1994 parameterization (34) followed by energy minimization with the Sander module of AMBER 9 (35), parm99 (36) force field, and the GB/ST/simulated annealing methodology (37) to represent solvent effects. A fully extended structure underwent 200 simulated annealing cycles of 100,000 molecular dynamics steps where system temperature was linearly raised from 10 to 1200 K (steps 1 to 5000), then kept constant at 1200 K (steps 5001 to 50,000), and finally linearly decreased down to 10 K (steps 50,001 to 100,000). A time step of 1 fs with no con-
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| Aggregation resistance based on structural homology | hCT | phCT | sCT | LAsCT |
|-----------------------------------------------|-----|------|-----|-------|
| CNT | C G N L S T C M L G T Y T D Q F N K F H F F P Q T A I G V G A P - NH₂ | C G N L S T C M L G T L T Q D F H K F H T F F P Q T N T G V G T P - NH₂ | C S N L S T C V L G K L S Q E L H K L Q T Y L A T N T G S G T P - NH₂ | C S N L S T C V L G K L S Q E L H K L Q T Y L A T N T G S G T P - NH₂ |

**FIGURE 1.** Primary structures of hCT, phCT, sCT, and LAsCT. The N-terminal disulfide bridge between positions 1 and 7 is boxed, and mutation sites are boxed and shaded. In phCT and LAsCT, mutated amino acids are in bold.

strains or restraints on bond lengths, a nonbonded cutoff of 1.6 nm, and a 0.05-fs time constant for heat bath coupling were used with all other parameters set at their default values. A semiparabolic penalty function with a force constant of 800 kJ mol⁻¹ nm⁻¹ was applied to interprotonic distances larger than upper limit values. Final structures were energy-minimized with 100 steps of steepest descent followed by a conjugate gradient method down to a gradient norm value < 4 × 10⁻² kJ mol⁻¹ nm⁻¹ both with the same penalty function used for experimental restraints in simulated annealing (restrained energy minimization) and under totally unrestrained conditions. The dynamic properties of phCT were evaluated with a fully unrestrained 1100-ps molecular dynamics simulation in methanol performed on the lowest energy structure following the procedure previously described for hCT and sCT (19) that included evaluation of angular order parameters S(φ) and S(ψ).

**RESULTS**

*Scanning hCT Sequence—*Calcitonin has been identified in many species, and sCT with only 50% sequence identity with hCT (Fig. 1) shows much greater biological potency than hCT and does not fibrillate. Both hCT and sCT in SDS assumes an amphipathic α-helix of different lengths in the central region and an extended C-terminal tail, but only in sCT do the structural elements interact (19). In particular, sCT presents a stable helical core involving residues Leu⁹–Leu¹⁹, whereas hCT takes up a shorter helix located in the Thr¹³–Phe¹⁹ region and shows a higher overall flexibility with respect to sCT (19). What will happen if the amyloidogenic hCT could take up the same three-dimensional structure of the aggregation-resistant sCT? Answering this question would certainly clarify some of the structural determinants that regulate hCT amyloidosis. Accordingly, we planned to induce in hCT the structure of sCT. As such, the first step could be the substitution of the hCT residues for the corresponding sCT residues and verification of the effect of such single/multiple substitutions on hCT amyloidosis. For this, we used an *in silico* approach applying the Waltz software (23). We first tested the software on hCT and sCT wild-type sequences, which were recognized as fibril-forming and aggregation-resistant according to reported experimental findings (11). In particular, for hCT, the Met⁸–Phe¹⁹ and the Ala²⁰–Val²⁹ regions were identified as amyloidogenic, and they include the central Asp¹⁵–Phe¹⁹ (38, 39) and the C-terminal region previously suggested as the fibril core (22, 39, 40).

Complete scanning of hCT with corresponding sCT amino acids (16 of 32 residues; Fig. 1) yields several thousands of mutants according to the formula \( n! / [k(n-k)!] \) where \( n = 16 \) and \( k = 1, 2, \ldots 16 \), the latter corresponding to the number of residues mutated simultaneously. The aggregation propensities of ~6000 sequences were analyzed, and the results are reported in Fig. 2. In general, we found a decrease in the aggregation propensity as the number of mutations incorporated into hCT approaches 16 (i.e. when hCT “becomes” sCT). Remarkably, with an occurrence >50%, substitutions in regions 11–13, 15–17, 19, 26–27, and 31 are predicted to decrease the aggregation propensity of hCT. To better define those regions, we limited our analysis to the Met⁸–Phe¹⁹ region, which contains eight mutations and includes the shortest hCT amyloidogenic sequence Asp¹⁵–Phe–Asn–Lys–Phe¹⁹ (38) and bears four mutations (underlined) (Glu¹⁵–Leu–His–Lys–Leu¹⁹ in sCT) (Fig. 1). We also examined the C-terminal region that contains hydrophobic patches involved in the fibrillation (22). Finally, we limited the number of changes to 5–6 amino acids (15–20% of the total number of residues) to minimize potential side effects. The analysis of ~2000 sequences of this reduced set of analogues did sharpen the above regions and with an occurrence >80% selected sites 12, 17, 26, 27, and 31. Interestingly, sites 17, 26, and 31 in sCT contain pivotal amino acids for the helix-tail interaction as indicated by NOEs observed between the βCH₂ of His¹⁷ and
the NH of Thr$^{31}$, the $\delta$CH$_2$ of Lys$^{19}$ and the NH of Asn$^{26}$, and the $\gamma$CH of Leu$^{19}$ and the NH of Asn$^{26}$ (41). According to the above data, we engineered the phCT analogue in which Asn$^{27}$, Ala$^{26}$, and Ala$^{31}$ were changed for the corresponding sCT residues His, Asn, and Thr, respectively; Ile$^{27}$ was substituted for Thr to increase the polarity of the C-terminal tail, and Tyr$^{12}$ was substituted for Leu to stabilize the N-terminal region of the central helix (19) (Fig. 1).

Estimation of the secondary structure from CD spectra in water (not shown) of hCT, sCT, and phCT by the k2d neural network algorithm (25) suggests a low helix percentage of 10, 15, and 9%, respectively. Because an important step of our work is the stabilization of the hCT structure, we used SDS as a medium because it is widely used to structure random coil polypeptides by stabilizing $\alpha$-helical and $\beta$-structures, depending on the concentration (42). Furthermore, because the mechanism for in vivo fibrillation may possibly be dissimilar from that taking place in dilute in vitro conditions (Ref. 43 and references therein), the presence of SDS was useful to simulate the heterogeneous environment of membranes and cellular matrix. The presence of SDS does not alter the aggregation tendency of hCT but does increase the fibrillation time: we have in fact observed the formation of insoluble aggregates in a 4-month-old sample of hCT in aqueous SDS stored at room temperature, whereas no evidence of aggregation was observed for a 12-month-old sample of sCT in SDS (44). In SDS (pH 7.4 and 310 K), a dominant $\alpha$-helix is clearly discernible in CD spectra of phCT and hCT as two minima with high ellipticity values are observed at 208 and 220 nm. We estimated the presence of 44% $\alpha$-helix for phCT, a value comparable with 50% predicted $\alpha$-helix for sCT but significantly higher than 19% estimated for hCT. These data suggest that phCT does assume a well-defined secondary structure; however, do the five mutations affect the fibrillation of phCT?

**Fibrillation Studies**—It is well known that at 1 mg/ml hCT self-assembles to form fibrils within 21 min, whereas sCT shows a fibrillation time $>$8 months (11). The kinetic process of phCT amyloid fibril formation in vitro was analyzed in comparison with other calcitonins and their 1:1 mixtures by using standard amyloid monitoring techniques.

A ThT binding assay for the fibrillation kinetic behavior is reported in Fig. 3. As expected, hCT (curve 1) exhibited a sharp increase in fluorescence values that reached the maximum at 42 h, suggestive of a rapid fibrillation process. On the contrary, phCT and sCT (curves 5 and 6, respectively) revealed low fluorescence values during the whole experiment time (860 h). These results indicate that, similarly to sCT, the phCT fibrillation process is still in the lag phase during the experiment course, implying a much slower tendency to form classical amyloid fibrils with respect to hCT. As a comparison, we also added LAsCT (curve 7), an sCT mutant with a longer central helix and no helix-tail interaction (45). It also showed low fluorescence values during the experiment with a resulting low aggregation tendency. The phCT mutant was also a potent inhibitor of wild-type fibrillation: the lag time for a 1:1 mixture of phCT and wild-type hCT (curve 4) was $\sim$500 h under our conditions, increasing the lag time with respect to the free hCT by a factor of $\sim$10. Such a significant increase is noteworthy especially considering the potential application in a therapeutic formulation of hCT. The final fluorescence intensity of the 1:1 mixture was also significantly reduced relative to wild-type hCT, being a factor of 5 lower. Similar results were also observed for the 1:1 mixture of hCT and sCT (curve 3) as well as for the 1:1 mixture of hCT and LAsCT (curve 2), although the latter mixture shows a lag time of $\sim$200 h with an increasing factor of $\sim$5. All 1:1 mixtures (curves 2, 3, and 4) used in Fig. 3 contained the same hCT concentration as that of the single hormones (curves 1, 5, and 7). This was done to avoid a reduction of the hCT concentration in the mixtures, which could generate a fluorescence decrease. However, a lower concentration of hCT in the mixtures did not affect the above conclusions (not shown). Comparable results have been reported for the islet amyloid polypeptide (amylin) (46), a peptide hormone responsible for amyloid formation in type 2 diabetes that belongs to the calcitonin family, and for the 40-amino acid amyloid $\beta$-protein (A$\beta$40), which shows an inhibitory effect in vivo (47) and in vitro (48, 49) on the fibrillogenesis of the 42-amino acid (A$\beta$42) alloform.

Because the final fluorescence intensity directly depends upon several factors involving ThT and the quantity of fibrillar material formed, (50), we confirmed the above findings by checking the soluble material found at the end of the incubation time (60 days). Amino acid analysis showed that 80 and 78% of the starting material for sCT and phCT, respectively, were still present in solution at the end of the incubation period compared with 16% of the starting material for hCT. Surprisingly, the amount of soluble material of the 1:1 phCT-hCT mixture was 60% of the starting material. The enhanced solubility of phCT and of the 1:1 mixture could have important implication for long term storage and bioavailability and eventually decrease the risk of undesired immunogenic responses related to aggregation (51).

The time course of the phCT, hCT, sCT, and phCT-hCT mixture fibril formation was also monitored by CD measurements at 1, 42, and 164 h since dissolution. After 1 h (Fig. 4A),
all calcitonins and the phCT-hCT (1:1) mixture had a predominant random coil conformation, indicated by a minimum at 205 nm. Such a monomeric component decreased gradually as a result of fibril formation, and after 42 h, corresponding to the sharp increase in fluorescence values (Fig. 3), hCT showed a reduced random coil signal with an increased ellipticity value at 205 nm and the appearance of a relative minimum at 218 nm, an indication of the presence of an \(\beta\)-sheet conformation (Fig. 4 B), whereas the other calcitonins preserve their structure. Even after 164 h, phCT and the phCT-hCT mixture exhibit a random coil spectrum similar to that of sCT (Fig. 4 C). Retention of the phCT and of the phCT-hCT mixture structure during the incubation time, along with the amino acid analysis and ThT binding assay, implies that substitution of specific amino acid residues provided the mutated hCT and the phCT-hCT mixture with a structural stability that prevents self-association and/or maturation into amyloid fibrils. After 860 h, we observed a slight decrease of the random coil signal for the phCT-hCT (1:1) mixture at 205 nm, reaching a \(\theta\) value of \(-2.8\), whereas the trace of phCT remained essentially unchanged. However, because at 860 h we could not register an accurate hCT spectrum, we decided to report the CD spectra variation as in Fig. 4 just to illustrate the changes occurring.

Transmission electron microscopy studies conducted as a function of time confirm that the mutations significantly affect fibril formation. hCT mature amyloid fibrils were detected after 5 h of incubation that rapidly grew into massive and crowded aggregates (Fig. 5 A). Similar aggregates could be observed for sCT (Fig. 5 B) but with a rare prevalence and only at the end of the incubation time (60 days). The phCT sample showed no signs of fibril formation (Fig. 5 C), and after 60 days, it exhibited the rare appearance of short amyloid fibrils, resembling “broken” fibrils less ordered and amorphous (i.e., non-fibrillar) in some parts. Images of the phCT-hCT 1:1 mixture illustrated that amorphous aggregates were observed after 22 days without any indication of the presence of fibril-like species (Fig. 5 D). All considered, the designed peptide phCT presents a stability comparable with the non-amyloidogenic sCT and is itself a powerful fibril inhibitor.
Physiological Activity—Calcitonin is known to activate adenyl cyclase to generate cAMP through interaction with the calcitonin receptor. We therefore tested the biological activity of phCT alone and the phCT-hCT (1:1) mixture with respect to hCT and sCT, performing a cell-based cAMP assay with a T47D cell line and monitoring the ability to recognize and activate specifically the hCT receptor endogenously expressed. This cell line primarily expresses the receptor hCT\(_{a}\) (formerly known as hCRT2) and a lesser amount of receptor hCT\(_{b}\) (formerly known as hCRT1) in an expression pattern similar to that observed in osteoclasts, the final target for hCT in the treatment of osteoporosis, especially in postmenopausal women (52). The stimulation of the calcitonin receptor was quantified by monitoring the intracellular cAMP levels before and after incubation of T47D cells with hCT, sCT, phCT, and the phCT-hCT (1:1) mixture. All of the peptides and the mixture stimulated cAMP accumulation between 340- and 400-fold over basal levels (2–5 pmol/5 \times 10^5 cells). hCT was found to have the lowest potency compared with sCT, phCT, and the phCT-hCT (1:1) mixture, stimulating cAMP production with an EC\(_{50}\) of 210 ± 62 pm, whereas phCT was 2-fold more potent than sCT and 5-fold more potent than hCT (p < 0.05). Interestingly, the potency of the phCT-hCT (1:1) mixture was similar to sCT and ~3 times more potent than hCT (p < 0.05). This increased efficacy observed for the engineered phCT and the phCT-hCT (1:1) mixture is likely to be a direct consequence of the reduced propensity to aggregate and increased availability (18). This possibility was further investigated by measuring the persistence of cAMP stimulation in T47D in the presence of the different peptides (Fig. 6).

CAMP levels were measured after stimulation with a 100 nm concentration of each peptide and the phCT-hCT (1:1) mixture in T47D cells previously subjected to a pretreatment with the different peptides (restimulated) as well as in cells that had not been exposed to such pretreatment. An additional group of samples corresponded to pretreated T47D cells that had not been subjected to a second incubation with the different calcitonin variants (recovered). The observation of similar CAMP levels in acutely stimulated and restimulated cells with all of the peptides and the mixture indicates that no significant receptor down-regulation has occurred during the chase period in all cases. phCT and the phCT-hCT (1:1) mixture seem to persist in stimulating the accumulation of CAMP more effectively than hCT and at the same level as sCT once the peptides are withdrawn from the media (Fig. 6). This finding can be related to longer term receptor activation as is the case for sCT and perhaps is associated with an increased facility of phCT and the phCT-hCT (1:1) mixture to interact with membrane lipids (53, 54).

Further characterization of the efficacy of the engineered phCT and of the mixture would require testing their biological activity in animal models, bioavailability through different administration routes, pharmacokinetics, and immunogenicity. Furthermore, different phCT-hCT ratios should be tested to uncover the lowest phCT percentage needed to allow a drastic reduction of the amyloidogenic properties of hCT and limit potential side effects. We are currently investigating some of these points, and the results will be reported in due course.

Solution Structure of phCT—The last open question is about the structure-activity relationship of phCT: do mutations induce in the engineered hCT the same structure of sCT? The detailed solution structure of phCT in SDS was obtained by NMR spectroscopy using TOCSY and NOESY spectra recorded at different temperatures to separate overlapping resonances. The secondary structure of phCT was delineated from qualitative analysis of the sequential (\(\alpha\)CH\(_{n-1}\),NH\(_{n+1}\) and NH\(_{n}\),NH\(_{n+1}\) )and medium range (\(\alpha\)CH\(_{n-1}\),NH\(_{n+1}\),1 < n < 4, and \(\alpha\)CH\(_{n}\),\(\beta\)CH\(_{n+3}\)) NOEs and from \(^{3}J\)\(_{HNH}\) coupling constants (Fig. 7A). In the region Ser\(^{5}\)–Phe\(^{22}\), strong NH\(_{n}\),NH\(_{n+1}\) NOEs and weak \(\alpha\)CH\(_{n}\),NH\(_{n+1}\) cross-peaks suggest the presence of an \(\alpha\)-helical structure. The presence of several unambiguous \(\alpha\)CH\(_{n}\),NH\(_{n+1}\),\(\beta\)CH\(_{n}\),NH\(_{n+1}\) , \(\alpha\)CH\(_{n}\),\(\beta\)CH\(_{n+3}\), and three \(\alpha\)CH\(_{n}\),NH\(_{n+1}\) cross-peaks together with \(^{3}J\)\(_{HNH}\) < 6 Hz led to the conclusion that the Ser\(^{5}\)–Phe\(^{22}\) region forms an \(\alpha\)-helix. In fact, the presence of an NOE between \(\alpha\)Ser\(^{5}\) and \(\beta\)Met\(^{8}\) indicates that the helix includes part of the N-terminal ring up to Ser\(^{5}\). This behavior has also been observed for sCT, whereas in hCT, no ring residue is part of the helix (19). Residues in the region 4–9 are important for the stabilization of the amphipathic \(\alpha\)-helix: in fact, two long range NOEs (NHLeu\(^{4}\)–\(\beta\)Leu\(^{7}\) and \(\beta\)Leu\(^{4}\)–NHLeu\(^{7}\)), observed at all mixing times, confirm close interaction between the ring and the helix (Fig. 7B). From Glu\(^{24}\) onward, the presence of strong NH\(_{n}\),NH\(_{n+1}\) NOEs and weak \(\alpha\)CH\(_{n}\),NH\(_{n+1}\) together with some characteristic \(^{3}J\)\(_{HNH}\) values suggests the existence of a sequence of turns. Most importantly, we identified five long range NOEs (between H2 and H4 of His\(^{17}\) imidazole ring and...
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A

FIGURE 7. A, amino acid sequence of phCT and diagrammatic representation of the short and medium range NOE connectivities observed in SDS micelles at 310 and 324 K. NOE intensities are indicated by the thickness of the bars. A square below the one-letter code indicates a $J_{\text{HN,\text{HN}}}$ coupling constant $< 5$ Hz measured for that residue, whereas a circle denotes $6 < J_{\text{HN,\text{HN}}} < 7$ Hz. A crossed square/circle indicates slow $^{1}H/^{2}H$ amide exchange. $B$, backbone superposition of the 20 phCT periodically sampled structures along the 1-ns unrestrained molecular dynamics. Structures were superimposed for pairwise minimum r.m.s.d. of the N, Ca, and C atoms of residues 2–21.

γCH$_{3}$ of Val$^{29}$, the γCH$_{3}$ of Thr$^{21}$ and γCH$_{3}$ of Thr$^{31}$, the H4 of the Phe$^{22}$ ring and the Hβ of Pro$^{32}$, and the Hβ of Phe$^{22}$ and the NH of Thr$^{31}$) as a consequence of the close interaction between the central helix and the C-terminal region. This interaction has been observed in sCT but not in native hCT (19). Therefore, the insertion of the five selected sCT amino acids into hCT is able to induce in phCT the planned folding back of the C terminus toward the central helix.

Evidence was found for the presence of isomers with a cis peptide bond at Pro$^{32}$. It was identified by an NOE between the α protons of Thr$^{31}$ and Pro$^{32}$, whereas the trans form was identified by the presence of an $\alpha$-$\delta_{i+1}$ NOE cross-peak. Usually, the relatively slow isomerization between cis and trans forms of the prolyl peptide bond induces additional cross-peaks for nearby residues. In phCT spectra, two Thr$^{31}$ cross-peaks were observed in the TOCSY fingerprint region with an estimated cis population of 19%.

On the basis of restraint violations, AMBER energies and PROCHECK parameters, 20 conformers were selected of 200 calculated structures (Fig. 7B), and the structural statistics are summarized in Table 1. phCT shows an α-helix spanning residue 4–21 detected in all selected structures with the C-terminal tail folded back toward the helix. An additional short $\alpha_{3}$ helix encompassing residues 25–27 is observed in five structures (residues 24–27 in one structure). A continuous O$_{\alpha}$-H$_{\text{HN}}$-H$_{\text{HN}}$ hydrogen bond pattern spanning the region 3–22 is observed in all bundle structures (except for two structures where it stops at residue 21), and most of them correspond to slowly exchanging amide protons (Fig. 7A). The slow exchange most likely indicates intramolecular hydrogen bond formation; in fact, it is unlikely that a slowly exchanging proton is buried in the interior of a biomolecule as small as hCT, although burial within the hydrophobic core of the micelle is also possible.

An $i$-$i+3$ locking between Phe$^{22}$ and Thr$^{35}$ is observed in 10 structures and corresponds to a type-I β-turn centered on Pro$^{32}$, Gln$^{34}$, whereas in 12 conformers, an intraresidue O–H−O bond is formed in Thr$^{21}$. Interresidue $i$-$i+4$ O–H−O bonds occur in all selected structures for Cys$^{7}$–Thr$^{11}$ and Leu$^{9}$–Thr$^{13}$. Although potentially disturbing for the α-helix, they only induce a slight deviation from the ideal α-helical pattern of the backbone H-bonds. A salt bridge between Asp$^{15}$ and Lys$^{18}$ was observed in all selected conformers, whereas Oδ1 of Asn$^{26}$ forms a bifurcated H-bond with Thr$^{4}$ HN and O–H−O hydrogen atom engages an H-bond interaction with Oγ Thr$^{4}$ in all structures. Complex networks of backbone-side chain and side chain-side chain H-bonds stabilize the folding of the C-terminal region, sometimes involving very long range interactions. In particular, the side chain of Asn$^{26}$ seems to “switch” among possible folds of the C terminus by forming different specific medium and/or long range H-bonds with residues ranging from Lys$^{18}$ to Thr$^{31}$. These features are completely independent from the application of experimental restraints as they also emerge in unrestrained calculations (19).

The phCT α-helix is amphipathic in nature. With the only exception of Val$^{29}$, the hydrophobic residues align on the same side of the peptide forming an “apolar strip” (Fig. 8, A and B, yellow), whereas the polar residues (gray) are all located at the opposite side, and both “strips” extend down to the C terminus.

Evidence was found for the presence of isomers with a cis peptide bond at Pro$^{32}$. It was identified by an NOE between the α protons of Thr$^{31}$ and Pro$^{32}$, whereas the trans form was identified by the presence of an $\alpha$-$\delta_{i+1}$ NOE cross-peak. Usually, the relatively slow isomerization between cis and trans forms of the prolyl peptide bond induces additional cross-peaks for nearby residues. In phCT spectra, two Thr$^{31}$ cross-peaks were observed in the TOCSY fingerprint region with an estimated cis population of 19%.

On the basis of restraint violations, AMBER energies and PROCHECK parameters, 20 conformers were selected of 200 calculated structures (Fig. 7B), and the structural statistics are summarized in Table 1. phCT shows an α-helix spanning residue 4–21 detected in all selected structures with the C-terminal tail folded back toward the helix. An additional short $\alpha_{3}$ helix encompassing residues 25–27 is observed in five structures (residues 24–27 in one structure). A continuous O$_{\alpha}$-H$_{\text{HN}}$-H$_{\text{HN}}$ hydrogen bond pattern spanning the region 3–22 is observed in all bundle structures (except for two structures where it stops at residue 21), and most of them correspond to slowly exchanging amide protons (Fig. 7A). The slow exchange most likely indicates intramolecular hydrogen bond formation; in fact, it is unlikely that a slowly exchanging proton is buried in the interior of a biomolecule as small as hCT, although burial within the hydrophobic core of the micelle is also possible.

DISCUSSION

We have successfully designed an aggregation-resistant hCT analogue by assuming a simple postulate: reproducing the three-dimensional structure of a non-amyloidogenic pep-
tide (sCT) into an amyloidogenic analogue (hCT) avoids fibril formation. By inserting in phCT (the engineered hCT variant) just five sCT residues (Fig. 1), we obtained a solution structure very similar to that of sCT. The two molecules have a remarkable similarity from the N-terminal ring to the end of the helix with the C-terminal region interacting tightly with the helical His\(^{17}\) side chain in phCT, whereas a looser interaction is observed in sCT (Fig. 8, C and D). It is important to recall that the five sCT residues inserted into hCT are not contiguous; furthermore, hCT and sCT share only 50% sequence homology with the N-terminal loop highly conserved and the central helix preserving only 6 residues of 16. Therefore, although the helical regions are superimposable (Fig. 8B), divergence in the packing of the side chains has to be expected. Moreover, the position of the N-terminal loop with respect to the central helix is affected by the fact that the loop

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**TABLE 1**

Structural statistics for bundle of 20 selected phCT structures

| Minimum (min) and maximum (max) values are shown in parentheses. |
|---|

| Experimental restraints |  |
|---|---|
| Intraresidue NOEs | 72 |
| Inter-residue sequential NOEs (\(|i - j| = 1\)) | 118 |
| Inter-residue medium range NOEs (\(1 < |i - j| \leq 4\)) | 48 |
| Inter-residue long range NOEs (\(|i - j| > 4\)) | 6 |
| Total | 244 |

| Restraint violations* |  |
|---|---|
| Sum of restraint violations (nm) | 0.44 ± 0.029 (min, 0.39; max, 0.50) |
| NOE distances with violations >0.01 nm | 1.01 ± 0.25 (min, 6; max, 16) |
| NOE distances with violations >0.02 nm | 0.055 ± 0.083 (min, 0; max, 3) |

| AMBER 94 energy (kJ mol\(^{-1}\)) | −3764.13 ± 26.65 |

| R.m.s.d. from ideal covalent geometry |  |
|---|---|
| Bonds (nm) | 0.00094 ± 0.00002 |
| Angles (°) | 2.48 ± 0.05 |

| R.m.s.d. from average structure (nm) |  |
|---|---|
| Selected backbone (Gly\(^{2–}\)–Thr\(^{21}\)) | 0.027 ± 0.009 |
| Selected heavy (Gly\(^{2–}\)–Thr\(^{21}\)) | 0.079 ± 0.012 |
| All backbone (Cys\(^{1–}\)–Thr\(^{21}\)) | 0.17 ± 0.061 |
| All heavy (Cys\(^{1–}\)–Thr\(^{21}\)) | 0.21 ± 0.063 |

| Procheck_NMR (G-factor and Ramachandran analysis) |  |
|---|---|
| G-factors |  |
| Overall | −0.5 ± 0.08 (min, −0.65; max, −0.36) |
| Dihedral | −0.16 ± 0.13 (min, −0.38; max, 0.09) |
| \(x_1–x_2\) | −1.28 ± 0.14 (min, −1.53; max, −1.01) |
| \(x_1\) only | −0.18 ± 0.13 (min, −0.56; max, 0.05) |
| Residues in the favored region (%) | 86.6 ± 5.55 (min, 76.0; max, 96.0) |
| Residues in the additional allowed region (%) | 11.0 ± 6.85 (min, 4.0; max, 24.0) |
| Residues in the generously allowed region (%) | 3.00 ± 2.87 (min, 0.0; max, 4.0) |
| Residues in the disallowed region (%) | 0.0 ± 0.0 (min, 0.0; max, 0.0) |

* No restraint violation larger than 0.029 nm was observed.

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**FIGURE 8.** Ribbon and stick representation of phCT structure parallel (A) and perpendicular (B) to the helical axis. The color code for residue polarity is as follows: gray, hydrophobic residues; yellow, polar residues; red, acidic residues; and blue, basic residues. C and D, superposition of phCT (magenta) and sCT (green). In both panels, His\(^{17}\) is shown with transparent van der Waals surfaces. Views C and D are obtained from A after 45° and 90° rotations about the horizontal axis, respectively.
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residues Leu⁴–Cys⁷ may also be part of the helix. This is well evident for sCT and LAsCT whose helices include residues 4–7 (19, 41, 45) but not for hCT in which no ring residue is part of the helix (19, 44). In phCT, the presence of an NOE between αSer⁴ and βMet⁸ indicates that the helix includes Ser⁴, whereas for sCT, the helix extends to Leu⁴. Such a difference justifies the slight difference observed in Fig. 8, C and D, for the loop orientation.

Selection of the residues was obtained in silico by using the Waltz software. By substituting in hCT the corresponding sCT residues, we analyzed a total of ~8000 mutated sequences identifying sites 12, 17, 26, 27, and 31 as the most relevant to prevent aggregation (Fig. 2). phCT showed increased stability in solution, and its aggregation propensity was found to be enormously decreased when judged against hCT and compared very well with sCT in the aggregation assays. Most importantly, the cAMP stimulation (Fig. 6) indicates that the redesigned variant has increased physiological activity with an activity comparable with sCT in the aggregation assays (16, 17).

We also found that phCT, sCT, and LAsCT are effective inhibitors of wild-type hCT amyloidosis (Fig. 3). In general, inhibitors should be able to block fibril progression during the lag phase by hampering the rapid growth of the aggregates. For hCT, analysis of the fibrillation time versus the protein concentration (56, 57) and NMR data (58) indicate that the protofibrils (the initial micellar critical nucleus) could be a helical bundle where the hydrophobic sides of the amphipathic helices interact with each other. Accordingly, the presence of an intercalating molecule such as phCT, sCT, or LAsCT could be considered as an efficacious separator that prevents amyloidogenic intermolecular interactions, therefore forming a heteronuclear (phCT-hCT) aggregate that does not evolve into a fibril. We have reported that sCT and LAsCT, but not hCT, are able to dimerize via the hydrophobic face of an amphipathic helix and that Leu-Leu interactions stabilize the aggregate, thus preventing fibril maturation (20). This together with the results here reported suggests that a “helical separator” in an hCT solution could block the fibril propagation process. A plausible model of fibril initiation by hCT can be proposed.⁴ The hCT fibrillation at acidic pH proceeds more slowly as compared with the neutral condition. Size exclusion chromatography at pH 3.3 and 7.4 indicates that sCT is present in solution as a stable dimer, whereas hCT is eluted as a monomer at pH 3.3 but is in equilibrium between dimer and monomer at pH 7.4. The structure of dimeric sCT is stabilized via Leu hydrophobic interactions of the helix, whereas in hCT, a sequence of turns is observed in the central region where the key leucines are substituted for aromatic residues (20). Such mutations have different effects: the stable sCT dimeric interaction prevents evolution toward aggregates, whereas the sequence of turns in the central region of the monomer is not sufficiently defined to stabilize the dimer and significantly limits the number of possible chain conformations. As such, it could provide an effective structural framework for the interaction of critical core residues located in the 15–19 region. Collision of short helical-like stretches has been suggested as a mechanism to initiate the folding pathway of GCN4p1 (59) and in the model of helical intermediates set up for amyloid formation in natively unfolded peptides (Refs. 43 and 60 and references therein). Such an interaction would favor the formation of the protofibrils, which should mature into fibrils during the lag phase. We have suggested that, for an essentially unstructured polypeptide chain like hCT, orientation could be driven by a hydrogen bond donor and the center of a benzene ring, which acts as a hydrogen bond acceptor (20, 61). The relevance of aromatic residues was postulated (62), predicted theoretically (63), and shown experimentally (64) to increase the rate of fibril formation. Accordingly, intermolecular hydrogen bonds have been reported to play an important role in the association of the hCT molecules (58).

At least 25 protein misfolding diseases of unrelated etiology are associated with amyloid fibril deposition, and in a “sequence-specific” view, definite regions of their amino acid sequences seem to drive the fibrillation process. In fact, in vitro fibril formation of globular proteins and of short peptides can be modulated by changes in the amino acid sequence (38, 65–68). A “structural” view of fibrillation suggests that a way to prevent it is to stabilize an α/β discordant helix (i.e. an α-helix that should form a β-strand according to secondary structure predictions (69)) in helical conformation. In unstructured polypeptides, partially folded helix-containing conformers have been suggested to be on the pathway to fibril formation (70). A question arises: how stable should the helix be to avoid fibrillation? The helix stability is an important point as, depending on its strength, α-helix stabilization may facilitate as well as inhibit fibril formation (71). Most likely an efficient stabilization should lock the molecule in the so-called “α-basin” (a free energy surface dominated by α-helical structures with minima of comparable free energies separated by low barriers (72)), avoiding the migration toward the “β-basin.” Such α-helix → β-sheet conversion going through progressively looser helical segments can be hampered by targeting either the intact α-helix with ligand molecules (73) or the structurally independent folding units (turnlike structures) present in the peptide (72, 74). The presence of SDS, used as a helix stabilizer, does lengthen the fibrillation time (the lag time of the onset of fibrillation) but does not abolish hCT aggregation. We have in fact observed an increase of the lag time from minutes to ~2 months for the formation of insoluble aggregates in an hCT-SDS solution ([SDS]/[hCT] ≡ 100; data not shown), whereas no evidence of aggregation was observed for a 12-month-old sample of sCT in SDS at the.

⁴ R. M. Vitale, G. Andreotti, P. Amodeo, and A. Motta, manuscript in preparation.
same concentration ratio (44). An analysis of the NMR restraints violations together with the angular order parameters \( S(\phi) \) and \( S(\psi) \) of hCT and sCT in SDS (19) indicates that hCT exhibits a higher flexibility, uniformly distributed along the polypeptide chain, including the helix, suggesting that hCT structures exist in a continuous distribution of similar inter-converting conformers endowed with high mobility. On the contrary, sCT structures are clustered in small numbers of families with a limited freedom for the central helix. Therefore, an efficient stabilization against fibrillation requires a well defined helix with no fluctuations around a “helical average” as for sCT.

The hCT and sCT differences in helix stability recall the differences between the Aβ42 and Aβ40 amyloid \( \beta \)-proteins (72). The Aβ40 local minima in the \( \alpha \)-basin have lower free energies than the \( \beta \)-basin minima, whereas Aβ42 shows in both basins local minima of comparable free energies. Therefore, the Aβ40 conformers populate the \( \alpha \)-regions more frequently, whereas the lower barriers for \( \alpha \)-helix \( \rightarrow \) \( \beta \)-sheet transitions imply a lower \( \alpha \)-helix percentage for Aβ42. This is confirmed by the \( \alpha \)-helix content of Aβ40 and Aβ42 (32 and 19%, respectively) with Aβ42 reaching its maximal value significantly sooner than Aβ40 (72).

As reported (38, 67, 68), an additional destabilizing factor is the presence of a region carrying the aggregation potential of the entire polypeptide chain, and in fact, the helix stabilization in hCT does not prevent aggregation. Are the sequence-specific and the structural factors independent? The present study seems to suggest that they both concur to amyloidogenesis, although the relative contributions need to be determined. We redesigned the amyloidogenic hCT by stabilizing the fluctuating central helix and by changing the amino acid pattern in fibrillogenic regions. We combined both aspects by “imposing” on hCT the three-dimensional structure of the non-amyloidogenic analogue sCT. Similarly to sCT, such a structure dramatically slowed the fibrillation kinetics of the modified hormone pHCT, which was still in the lag phase at the end of the experiment (860 h), therefore implying a much slower tendency to form classical amyloid fibrils with respect to hCT. The pHCT mutant was also a potent inhibitor of wild-type fibrillation as the lag time for a 1:1 mixture of pHCT and wild-type hCT increased by a factor of \( \sim 10 \). Such a significant increase is noteworthy especially considering the potential application in therapeutic formulation of hCT. The final fluorescence intensity of the 1:1 mixture was also significantly reduced relative to wild-type hCT, being a factor of 5 lower. Similar results were also observed for the 1:1 mixture of hCT and sCT (Fig. 3, curve 3) as well as for the 1:1 mixture of hCT and LAsCT (curve 2), although the latter mixture shows a lag time of \( \sim 200 \) h with an increasing factor of \( \sim 5 \). All 1:1 mixtures (curves 2, 3, and 4) used in Fig. 3 contained the same hCT concentration as that of the single hormones (curves 1, 5, and 7). This was done to avoid reduction of the hCT concentration in the mixtures, which could generate a fluorescence decrease. However, a lower concentration of hCT in the mixtures did not affect the above conclusions (not shown).

Our results could be in line with the formation of mixed oligomers (49) whose stoichiometry and mechanism(s) need to be elucidated. Furthermore, the minimal concentration of the inhibitory peptide in the mixture with hCT able to prevent further oligomerization should also be determined. Finally, our data together with those for the islet amyloid polypeptide and Aβ40 may favor a new strategy for modulating fibril formation in a variety of human diseases and the design of aggregation-resistant analogues acting as inhibitors of amyloidogenesis. This approach could also be used to facilitate the general redesign of polypeptide therapeutics to produce a much higher resistance to aggregation. The major aims of such an approach would be to optimize production, formulation, posology, and shelf-life for polypeptide drugs.

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