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NMR Characterization of Monomeric and Oligomeric Conformations of Human Calcitonin and Its Interaction with EGCG

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Abstract

Calcitonin is a 32-residue peptide hormone known for its hypocalcemic effect and its inhibition of bone resorption. While calcitonin has been used in therapy for osteoporosis and Paget's disease for decades, human calcitonin (hCT) forms fibrils in aqueous solution that limit its therapeutic application. The molecular mechanism of fiber formation by calcitonin is not well understood. Here, high-resolution structures of hCT at concentrations of 0.3 mM and 1 mM have been investigated using NMR spectroscopy. Comparing the structures of hCT at different concentrations, we discovered that the peptide undergoes a conformational transition from an extended to a β -hairpin structure in the process of molecular association. This conformational transition locates the aromatic side chains of Tyr12 and Phe16 in a favorable way for intermolecular π - π stacking, which is proposed to be a crucial interaction for peptide association and fibrillation. One-dimensional ¹H NMR experiments confirm that oligomerization of hCT accompanies the conformational transition at 1 mM concentration. The effect of the polyphenol epigallocatechin 3-gallate (EGCG) on hCT fibrillation was also investigated by NMR and electron microscopy, which show that EGCG efficiently inhibits fibril formation of hCT by preventing the initial association of hCT before fiber formation. The NMR experiments also indicate that the interaction between aromatic rings of EGCG and the aromatic side chains of the peptide may play an important role in inhibiting fibril formation of hCT.

Keywords

amyloid peptide; calcitonin; oligomerization; conformation; EGCG

Introduction

Calcitonin is a 32-amino-acid peptide hormone with an N-terminal disulfide bridge between Cys1 and Cys7 and a C-terminal proline amidated residue (Fig. 1). Secreted by the thyroid in response to elevated serum calcium levels,^{1,2} calcitonin acts to reduce blood Ca²⁺ levels and to reduce bone resorption by inhibiting the activity of osteoclast cells.^{3–6} Because of its

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Supplementary Data

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ability to limit bone resorption, calcitonin has been used as a treatment of osteoporosis for more than two decades.⁷ However, the therapeutic application of human calcitonin (hCT) has been limited by its tendency to assemble into inactive, fibril-like aggregates in aqueous solution.^{8–10} Since amyloid fibril formation is also observed in many other peptides associated with degenerative disorders, including Alzheimer's disease, Parkinson's disease and type II diabetes, a comprehensive understanding of the fibrillation process of amyloid peptides and the development of fibril inhibitors are desirable not only for the further improvement of medicinal efficiency of calcitonin but also in general for the development of treatment for amyloid-related diseases.

The structure of calcitonin fibers and other oligomeric species have not been determined at high resolution at the molecular level. On a mesoscopic scale, electron microscopy studies have shown that hCT fibers are approximately 80 Å in diameter with the cross- β -sheet architecture common to amyloid fibers.¹¹ At the molecular level, the assembly process of hCT monomers into amyloid fibers has been examined in some detail using NMR and other methodologies. Fibrillogenesis of hCT is a pH-dependent process with rapid association into antiparallel β -sheets at pH 7.5 and slower association into antiparallel and parallel β -sheets at pH 3.3,^{12–14} a switch that appears to be controlled by the ionization states of Asp15, Lys18 and His20.^{13,14} The residues in the central region of hCT are sufficient for fiber formation, as a truncated pentapeptide fragment of hCT from Asp15 to Phe20 (DFNKF) forms fibrils similar to those formed by the intact peptide.^{15,16} Kinetic studies have suggested that hCT amyloidogenesis is a multistep process, with non-fibrillar intermediates playing a key role in the early stages of amyloidogenesis.^{12,17,18} A time-dependent solution NMR study showed that peaks from residues in the central region (M8–P23) of the peptide broaden and disappear much faster than those in the C-terminus during fibril formation, suggesting that these residues are involved in the formation of an intermediate before assembly of the final amyloid fiber.¹⁹ The existence of an intermediate state of hCT during fiber formation is supported by transmission and attenuated total reflection FTIR (Fourier transform infrared spectroscopy) experiments that showed the simultaneous increase of α -helix and β -sheet components during fibril formation.¹⁸ Solid-state NMR studies suggest a localized α -helix structure around G10 at pH 3.3, which is slowly converted into a β -sheet structure as the fibrils are formed.^{12,14} However, a high-resolution structure of an intermediate conformation has not been reported.

Knowledge on the oligomerization process may be utilized in the design of drugs to stop it. Fibrillation of amyloid peptides sometimes can be inhibited by small molecules through different pathways: small molecules including dopamide,²⁰ calmidazolium chloride,²¹ hydroxyindole derivatives²² and sulfated triphenyl methane derivative acid fuchsin.²³ (–)-Epigallocatechin 3-gallate (EGCG), a polyphenol compound obtained from green tea, in particular, has raised much interest due to its strongly inhibitory effect on fibrillogenesis and its low toxicity. So far, it has been reported that EGCG efficiently inhibits fiber formation of α -synuclein,^{24–26} A β ,^{26,27} huntingtin,²⁸ IAPP²⁹ and MSP2.³⁰ However, the mechanism of the inhibition has not been well established due to the complexity of the interaction between EGCG and amyloid peptides, which may involve an initial non-covalent interaction, a covalent modification of the peptide^{26,30} and auto-oxidation and quinone formation of EGCG.³¹ As EGCG provides a promising lead for further development of amyloid fibril inhibitors, a comprehensive understanding of the inhibitory mechanism at the atomic level is desirable.

In this study, we have investigated the oligomerization of hCT by examining the influence of peptide concentration and amyloid inhibitors on the conformation of hCT by NMR spectroscopy. Toward this end, we have solved high-resolution structures of hCT at two different concentrations using NMR spectroscopy. Our results indicate a site-specific

conformational transition of hCT from an extended structure to a β -hairpin in the central region (Y12–F19) of the peptide upon an increase in hCT concentration from 300 μ M to 1 mM, consistent with a change in peptide oligomerization state detected by diffusion-ordered NMR. To determine the effect of amyloid inhibitors on the oligomeric conformation of hCT, we investigated the inhibitory effect of EGCG on hCT fibril formation. Our results indicate that EGCG efficiently inhibits fibril formation of hCT by preventing the initial oligomerization step. The interaction between EGCG aromatic rings and peptide aromatic side chains may play an important role in preventing hCT amyloid fibril formation.

Results

Oligomerization of hCT

To examine the oligomerization state of hCT in solution, we initially acquired one-dimensional (1D) ^1H NMR spectra at two concentrations—0.3 mM and 1 mM. A broad peak observed at -0.1 ppm in the 1-mM hCT sample was absent in the 0.3-mM hCT sample (Fig. 2a, inset). This peak is commonly found in the spectra of amyloidogenic proteins, and its appearance is consistent with a species in which at least some of the aliphatic protons are significantly protected from solvent and thus shifted to the highfield region of the spectrum (Fig. 2a).^{32–35} However, the relatively narrow dispersion of resonances in other regions of the ^1H spectra suggests that the peptide is at least partially unfolded at both concentrations (see Fig. 2). This difference is consistent with a heterogeneous sample containing at least two species, one significantly folded and the other largely unfolded. To confirm this hypothesis, we performed pulsed-field gradient (PFG) NMR diffusion experiments to estimate the hydrodynamic radius of peaks that likely represent folded and unfolded species (see Fig. 3). A hydrodynamic radius of 1.57 nm was obtained for both samples for peaks between 8.6 and 7.4 ppm, suggesting that these peaks correspond to a monomeric, significantly unfolded peptide at both concentrations. For the peak at -0.1 ppm, the radius of gyration could not be measured precisely. However, the insensitivity of the peak intensity to the gradient strength suggests a very slowly diffusing oligomeric species at least 50 nm in diameter, in agreement with previous studies on other amyloidogenic proteins.^{34,35} Overall, the ^1H spectrum in the 0.3-mM sample is consistent with hCT being exclusively in the monomeric state and the monomer coexisting with large oligomeric species at 1 mM hCT concentration. However, the only contribution of the large oligomeric state to the ^1H spectra of the 1-mM sample is the small peak near -0.1 ppm, as other resonances originating from the large oligomeric state are apparently broadened beyond detection.

Concentration-dependent NOE patterns of hCT

The partial protection of the amide hydrogen suggests that hCT is at least partially structured at pH 2.9. We confirmed this result by recording two-dimensional (2D) $^1\text{H}/^1\text{H}$ total correlated spectroscopy (TOCSY) and nuclear Overhauser enhancement spectroscopy (NOESY) spectra of samples of hCT at 0.3 mM and 1 mM concentrations. The spectra showed relatively good resolution at both concentrations; all intraresidue nuclear Overhauser enhancements (NOEs) between backbone amide protons and α protons could be identified except for those originating from C1. Figure 4 shows the $\text{H}^\alpha\text{--H}^\text{N}$ region of the 2D NOESY spectrum at 1 mM hCT concentration. Figure 5 shows the NOE connectivities obtained for both concentrations.

Table 1 summarizes the NOE constraints obtained for both concentrations. A far larger number of NOEs were observed for the 1-mM concentration compared to the 0.3-mM sample. For the 1-mM sample, a total of 291 NOE constraints could be detected, while only 131 could be determined for the 0.3-mM sample. This difference persisted even after the signal was normalized for the difference in concentration between the two samples. The

relative increase in the number of NOEs in the 1- mM spectra was not uniform but was largely concentrated in the H^N - $H^{\text{aliphatic}}$ region of the 2D NOESY spectrum. The uneven distribution can be seen in Fig. 6, which shows the superposition of the H^N - $H^{\text{aliphatic}}$ regions of the NOESY spectra at both concentrations normalizing for the concentration difference. Normalization is done by reducing the contour level of the 0.3- mM spectra by 3.3 times compared with the 1- mM spectra in Sparky. Several additional NOEs are particularly prominent, mostly those between side-chain protons of residue i and amide protons of residue i or $i+1$ in the central region of the peptide, for example, those between M8H γ 2- H^N , L9H δ - H^N , C7HH β 3-M8 H^N , T11Q γ 2-Y12 H^N , T13Q γ 2-Q14 H^N , D15H β 3-F16 H^N , F19H β 3-H20 H^N , P23H β 2-Q24 H^N and Q24H β 2-T25 H^N (see Fig. 6). In addition, $^1H/^1H$ NOEs $d_{\alpha N}(i,i+2)$ between S5 and C7, between G10 and Y12 and continuously from D15 to H20 were observed in the 1- mM hCT sample. These NOEs are absent in the 0.3- mM hCT sample, which suggests a more constrained structure in the central region of the peptide at the higher concentration. A $d_{\alpha N}(i,i+2)$ $^1H/^1H$ NOE connectivity between D15 and N17 is characteristic of a turn structure around these residues.³⁶ The presence of a type II β -turn in the 1- mM sample is also well supported by the intense $d_{NN}(i,i+1)$ NOEs between F16 and N17 and medium- and long-range interresidue NOEs from either side of the turn between residues T13/N17, Q14/N17 and T13/F19. These medium- and long-range NOEs are listed in Table 2.

From these NOEs, structural models of hCT could be constructed for both samples (see Fig. 7). For the 0.3- mM sample, hCT is largely unstructured, in agreement with the paucity of NOEs detected at this concentration. In the 1- mM sample, on the other hand, a well-defined β -hairpin structure is present in the central region of the peptide from Y12 to F19. The hairpin structure is mainly constrained by 19 medium-range and 2 long-range NOEs observed between the two strands (see Fig. 7c for a cartoon depiction), especially from residues T13 and Q14 on the N-terminal side of the hairpin to residues N17 and F19 on the C-terminal side. A β -turn from Q14 to N17 separates the strands of the hairpin. The β -turn is further stabilized by additional hydrogen bonds originating from side chains within the turn (red broken lines in Fig. 7c). In all the energy-minimized structures, the aromatic residues Y12 and F16 within the turn region are consistently oriented so that the planes of the aromatic rings are parallel with the face of the hairpin, presumably favoring peptide stacking through intermolecular π - π interaction. While the 20 energy-minimized backbone structures superimpose well at the central region of the peptide from Y12 to F19 in the 1- mM sample, the N- and C-terminal residues are largely disordered in both samples.

EGCG inhibits the formation of oligomeric species of hCT

Having characterized a possible oligomeric species of hCT, we next examined the effect of EGCG on hCT oligomerization. We first confirmed that EGCG inhibits amyloid fiber formation by hCT, similar to what has been observed for other amyloidogenic proteins.^{26,29,30,37-40} To observe the effect of EGCG on hCT fibrillization, we incubated samples of 100 μ M hCT with and without 500 μ M EGCG at pH 7.4 for 36 h. In the absence of EGCG, hCT aggregates to form a dense network of amyloid fibers (Fig. 8a). On the other hand, when hCT was incubated with a 5-fold excess of EGCG under the same condition, fibers or fiber-like aggregates were not observed (Fig. 8b). The black dots observed in the hCT/EGCG sample match those observed in the control sample containing EGCG only (see Supplementary Fig. S1), suggesting that they correspond to EGCG aggregates. The absence of amyloid fibers in the hCT/EGCG sample therefore implies that the fibrillation of hCT was efficiently inhibited by interaction with EGCG.

Hydrogen/deuterium (H/D) exchange experiments were carried out to further study the effect of EGCG on the oligomerization process of hCT. The time course of H/D exchange of hCT amide protons with and without EGCG is shown in Fig. 9. A comparison of the two

sets of 1D spectra shows that the H/D exchange rates of amide protons of hCT are much faster in the presence of EGCG. In the absence of EGCG, most of the amide peaks could still be observed after 48 h of deuterium exchange, although the intensity and resolution of the peaks decreased significantly (Fig. 9a). In contrast, the peaks from the amide protons were almost disappeared within 15 min in the EGCG-treated hCT sample, indicating near complete exchange in this sample. Since monomeric hCT is almost completely unstructured (see Fig. 7a), the faster exchange rate is likely to arise from the elimination of oligomeric species of hCT, which have a substantial degree of intermolecular hydrogen bonding (see Fig. 2b). This conclusion is further supported by the absence of the oligomer peak at around -0.1 ppm in the EGCG-treated hCT sample (Fig. 9c), which also suggests the inhibition of the growth of large oligomer species by EGCG.

Mechanism of EGCG binding to hCT by NMR experiments

To elucidate the binding mechanism of EGCG to hCT, we recorded NMR spectra of hCT in the absence and presence of a 5-fold molar excess of EGCG. A shifting of the resonances of both hCT and EGCG indicated direct binding between EGCG and non-fibrillar forms of the peptide (amyloid fibers are not detected in these experiments as the long rotational correlation time of the fiber broadens the signal beyond detection). The shifting of the resonances of hCT upon the addition of EGCG was investigated in more detail by 2D NMR (see Fig. 10). A superimposition of the $^1\text{H}/^{15}\text{N}$ heteronuclear multiple quantum coherence (HMQC) spectra of hCT with and without 2 molar equivalents of EGCG shows an almost universal shift of the resonances upon EGCG binding (Fig. 10a), indicating that EGCG binding to the monomer is not localized on specific sites within the peptide but is rather largely delocalized, in agreement with previous experiments on α -synuclein.²⁶

The chemical shift changes of the amide and α protons of hCT occurring upon EGCG binding are summarized in Fig. 10b, calculated from the $^1\text{H}/^{15}\text{N}$ HMQC and NOESY spectra, respectively. The amide protons of residues from L9 to the C-terminus are shifted to high field except for T11 and D15, while the N-terminal residues, where there is a disulfide loop from C1 to C7, are shifted to low field only slightly. The magnitude of the changes suggests that EGCG largely binds in the central and C-terminal regions of the peptide. The general high-field shift observed is most likely due to the shielding effect of the aromatic rings of EGCG. Interestingly, amide protons of T11, D15 and T21 show either “abnormal” low-field shifts or a negligible shifts differing from the adjacent residues.

Although changes are apparent throughout the spectrum when EGCG binds to hCT, aromatic side chains appear to play a particular role. The residues with the most significant H^α chemical shift differences ($\delta > 20.1$ Hz, compared to the mean value of 9.2 Hz) are almost all aromatic residues (Y12, F16, F22 and A31), suggesting a close spatial proximity between these residues and EGCG. The resonances from protons on aromatic side chains also show significant chemical shift perturbations induced by EGCG (Fig. 10c), which might be due to a π - π stacking interaction between aromatic rings of EGCG and the side chains of these residues. Notably, the amide protons showing atypical shifts upon EGCG interaction (T11, D15 and T21) directly precede the aromatic residues Y12, F16 and F22. This pattern is consistent with parallel stacking of aromatic rings, which should cause a deshielding effect on the α protons in the adjacent residues as they lie perpendicular to the aromatic rings.

Discussion

Our study reveals that a conformational change in hCT accompanies peptide association and oligomerization. At a concentration of 0.3 mM, hCT adopts a flexible and extended structure. A small degree of structure is present in the form of a loose turn from D15 to F19 in the central region of the peptide. However, the peptide is almost entirely unstructured at

this concentration, a fact supported by the PFG results showing a hydrodynamic radius of 1.57 nm, which is similar to that of completely denatured peptides but is larger than most amyloidogenic peptides of this length that are typically partially folded.^{34,35,41} Both IAPP and A β _{1–40}, for example, have low-lying helical states that are believed to nucleate amyloid formation.⁴² However, NOEs consistent with a helical structure were not detected for hCT at pH 2.9, as has been proposed for hCT oligomers in acidic solution and as a low-lying excited state of the monomer at neutral pH.^{12,14} The absence of a helical structure in the monomer is consistent with the finding that metastable secondary structure correlates with amyloidogenicity of the peptide,^{43–45} as fiber formation is strongly delayed in hCT at an acidic pH compared to neutral pH.¹⁹ At this concentration, the peptide does not form large oligomers during the experimental time period according to the ¹H NMR spectra.

At a higher hCT concentration of 1 mM, many more NOEs could be detected in the sample after normalization, particularly in the central region of the peptide from G10 to H20. From these additional NOEs, a new structure that is significantly more ordered than the monomeric structure obtained for the 0.3- mM sample could be constructed. The loose turn found in the 0.3- mM structure is transformed into a well-defined type II β -turn between Q14 and N17, and the central region of the peptide adopts a well-defined extended conformation from Y12 to H20, which superimposes perfectly among the 20 energy-minimized structures. The C- and N-termini, including the disulfide ring, are unstructured at both concentrations. The hairpin conformation is similar to that previously obtained for hCT in 85% dimethyl sulfoxide and 15% H₂O; however, the turn was located in a different position in this study (N17–H20), possibly because of a different ionization state of the peptide.⁴⁶

The change in the conformation of hCT as the concentration is increased implies that the structure obtained at the 1- mM concentration is of an oligomeric species while the 0.3- mM structure is solely that of a monomer. On the other hand, the hydrodynamic radius of hCT in the 1- mM sample calculated from most of the peaks in the 1D ¹H spectrum nearly exactly matches that of the 0.3- mM sample, suggesting that the 1- mM sample still consists largely of monomeric peptide. However, the 1D ¹H and diffusion experiments (Fig. 2) also indicate large oligomer species in coexistence with the monomer in the samples at 1 mM but not 0.3 mM concentration. Amyloidogenic peptides frequently form micellar-type aggregates at intermediate peptide concentrations,^{47–51} which can undergo exchange with the monomeric peptide.^{34,52} Such micelles have been proposed as intermediates for calcitonin fibrillization.¹² Therefore, the most likely explanation is that the additional NOEs observed in the 1- mM sample represent transfer NOEs from a large, mostly NMR-invisible species in rapid exchange with the monomeric peptide. Under particular conditions (k_{off} being fast relative to the T1 relaxation time of the monomer/oligomer complex and NOE mixing time), the “memory” of the conformation of the hCT in the oligomer can be effectively carried over into the NOESY spectra in the form of additional NOE cross peaks, even though the oligomer itself is not visible to NMR.⁵³ Note that transfer NOEs are typically observed when the binding partner (in this case, the oligomer) is in large excess, in agreement with the similarity of 1D ¹H spectra.⁵³

The association of aromatic side chains has been proposed to be a driving force for fibrillization of hCT.^{15,17} For instance, molecular dynamic studies have shown that a five-residue segment of hCT starting from D15 (DFNKF) forms high-ordered fibrils similar to the intact peptide.⁵⁴ In contrast, the phenylalanine-to-alanine analogue (DANKA) does not form amyloid fibrils at all, implying an important role of aromatic interactions in peptide association.¹⁵ In the hairpin structure of the 1- mM sample, the planes of aromatic rings on the side chains of Y12 and F16 are primarily parallel with the hairpin plane (Fig. 7c and d),

which potentially favors the association of the peptide along the normal of the hairpin plane by the π - π interaction between the phenyl rings from adjacent peptides.

The apparent importance of aromatic interactions in stabilizing oligomeric conformations of hCT is shown by the interaction of hCT with EGCG. EGCG acts as fibril inhibitors of several amyloid peptides by stabilizing their oligomers in an off-pathway conformation that prevents their conversion to a toxic on-pathway oligomer intermediate,^{24,26,30} although apparent exceptions exist.^{29,38,55} Our NMR results indicate that EGCG inhibits fibrillation of hCT by stabilizing the monomeric form of hCT in a largely unstructured conformation and by preventing the oligomerization of hCT. In the ¹H NMR spectrum, we did not observe the slowly diffusing oligomer peak of EGCG-treated hCT around -0.1 ppm, which suggests the absence of large oligomer species in the EGCG-treated sample. In addition, the amide peaks of EGCG-treated hCT were quenched before those of the untreated sample in H/D exchange experiments. This suggests that EGCG-treated hCT is in a more solvent exposed environment, most likely due to stabilization of the unstructured monomeric form of the peptide. While the NMR spectra indicate that the interaction of EGCG affects the chemical shift values of most of the residues (Fig. 10), the α protons of the aromatic side chains Y12, F16 and F22 show more significant chemical shift perturbations by EGCG (Fig. 10b). In addition, the amide protons of the residues preceding the aromatic residues (T11, D15 and T21) show atypical low-field chemical shift changes upon interaction with EGCG, suggesting a ring current effect on these residues. From these results, it is reasonable to conclude that π - π interactions between EGCG and Y11, F16 and F22 are involved in the association of EGCG with hCT. If intermolecular π - π interactions play an important role in amyloid formation of hCT, it can be expected that the competing interaction of the aromatic rings of EGCG for these side chains blocks potential sites for peptide association and prevents oligomer formation.

It should be noted that, despite apparent superficial similarities with models of β -sheet amyloid oligomers, the oligomer structure reported here would require a conformational change before it can be incorporated into the hCT amyloid fiber. X-ray crystallography and solid-state NMR measurements have suggested that individual protein molecules in amyloid fibers typically adopt a common hairpin-type structure with the side chains of residues forming a tight interface known as a “steric zipper” between the two β -strands.⁵⁶⁻⁵⁸ Intermolecular association of the proteins along the fiber axis is responsible for fiber formation.

The tight packing of side chains in the steric zipper interface necessarily gives rise to a high number of NOEs between the β -strands.⁵⁹ NOEs of this type were not observed in the 1-mM oligomer sample except in the immediate vicinity of the type II turn. In addition, the loop separating the β -strands in the hairpin is typically ~10 residues in length.^{57,58} A smaller loop, such as the type II turn observed in the 1-mM oligomer sample, requires either the side chains to be pointed away from the plane of the hairpin or the strands to be separated in space. Either conformation is not likely to be conducive to the formation of the steric zipper interface essential for amyloid formation. For this reason, the oligomer conformation reported here is unlikely to be a direct nucleus for amyloid fiber formation. Measurements of the 1D ¹H spectra in real-time and solid-state NMR spectroscopy suggest that α -helical oligomers instead may be involved as a direct intermediate of fiber formation at both low and neutral pH.^{12,19} Conformational changes are often observed in amyloidogenic peptides following peptide association in which non-fibrillar oligomers such as those observed here are rapidly formed and then slowly converted into amyloid fibers (the nucleated conversion model).⁶⁰ Further investigation is needed to determine if the oligomer structure determined here is on- or off-pathway for amyloid formation.

Materials and Methods

Materials

EGCG (95% purity) was purchased from Sigma and used without further purification. Concentrated (25 mM) stock solutions of EGCG were prepared by dissolving EGCG in N₂ flushed water. hCT was synthesized by Fmoc chemistry using an Applied Biosystems 431A peptide synthesizer and Fmoc amino acids purchased from Watanabe Chemical Industries (Hiroshima, Japan). An amide resin (Applied Biosystems, Inc., Foster City, CA) was used for the formation of the C-terminal amine. After deprotection and cleavage from the resin, the peptides were purified by reversed-phase HPLC using a mobile phase of water and acetonitrile containing 0.05% trifluoroacetic acid. The disulfide bridge between Cys1 and Cys7 was formed overnight by air oxidation of dilute (0.5 mM) hCT solution in 0.1 M sodium acetate (pH 8.0–8.5) in the presence of 6 M urea to prevent fibrillization.⁶¹ After an Ellman test confirmed the presence of the disulfide bond,⁶² the oxidation reaction was stopped by adding an acetic acid solution. The oxidized product was then repurified by HPLC. After purification, the peptide was lyophilized from aqueous solution in 40% acetonitrile (used to maintain the monomeric conformation) and stored at –4 °C.

H/D exchange

Samples for H/D exchange experiments were prepared by first dissolving lyophilized hCT in phosphate buffer [1 mM hCT, 7% D₂O and 50 mM NaCl (pH 2.9)] to a concentration of 1 mM in the absence and presence of 4 molar equivalents of EGCG. Amide proton/deuteron exchange was initiated by exchanging the original phosphate buffer into equivalent deuterated buffer using a Zeba Spin Desalting Column from Thermo Scientific. We recorded 32 transients for each ¹H spectrum before H/D exchange and for the indicated time durations after the H/D exchange.

Electron microscopy measurements

Samples for electron microscopy were prepared by first dissolving lyophilized hCT in phosphate buffer (pH 7.3, 20 mM and 100 mM NaCl) at a peptide concentration of 100 μM and incubating at 25 °C for 36 h in the absence and presence of 3 molar equivalents of EGCG. Aliquots of 10 μL samples were deposited on a 400-mesh copper grid and incubated for 2 min, washed with distilled water twice and negatively stained with 10 μL of 2% (w/v) uranyl acetate for 1.5 min and dried. Observations were performed on a Philips CM-100 electron microscope.

Sample preparation for NMR experiments

A few minutes before starting the NMR experiments, samples were prepared by dissolving the lyophilized hCT peptide in 20 mM phosphate buffer (pH 7.4 or pH 2.9) containing 50 mM NaCl to a final concentration of 0.3 or 1 mM. The stability of the sample over the time course of the experiment was tested by obtaining a series of ¹H chemical shift spectra over a period of several days. The high degree of similarity of the spectra confirmed the stability of the sample at an acidic pH of 2.9 over the time course of the experiment. However, the sample at pH 7.4 rapidly aggregated at the concentrations with the solution becoming visibly turbid and with the intensity of the signal rapidly diminishing as time progressed, in agreement with previous reports.¹⁹ Accordingly, all NMR experiments were performed at pH 2.9.

NMR spectroscopy

All NMR experiments were performed on a Bruker *A VANCE* 900-MHz spectrometer at 25 °C. Samples were prepared by dissolving lyophilized hCT in phosphate buffer (pH 2.9, 7%

D₂O and 50 mM NaCl) at peptide concentrations of 0.3 mM and 1 mM. 2D TOCSY and NOESY spectra were obtained for structure determination. TOCSY spectra were recorded using 256 t_1 experiments, 80 ms of mixing time and 4 scans, while NOESY spectra were obtained using 512 t_1 experiments, 300 ms of mixing time and 16 scans. The proton frequency for each experiment was set on water resonance (4.7 ppm). The spectra were referenced relative to 4,4-dimethyl-4-silapentane-1-sulfonic acid (DSS). Spectra were processed using TOPSPIN and analyzed using Sparky. The NOE cross peak assignments were obtained by an iterative procedure using a combination of manual and automatic approaches.

Proton diffusion NMR measurements were carried out using the STE (s $\acute{a}$ mulated echo) PFG pulse sequence with squared gradient pulses of constant duration (5 ms) and a variable gradient amplitude along the longitudinal axis.⁶³ Typical acquisition parameters used in NMR experiments were as follows: a 90° pulse width of 23 μ s, a spin echo delay of 10 ms, an STE delay of 150 ms, a recycle delay of 5 s, a spectral width of 10 kHz and 4048 data points. A saturation pulse centered at the water frequency was used for solvent suppression. Radio frequency pulses were phase cycled to remove unwanted echoes. All spectra were processed with an exponential multiplication factor equivalent to a 5-Hz line broadening prior to Fourier transformation and were referenced relative to DSS. The gradient strength was calibrated ($G=3.28$ T/m) from the known diffusion coefficient of HDO in D₂O at 25 °C ($D_0=1.9\times 10^{-9}$ m²/s).⁶⁴ The diffusion coefficients were determined from the slope of a log plot of the intensity as a function of gradient strength using the Stejskal–Tanner equation.⁶⁵ The hydrodynamic radius was then calculated from the diffusion coefficient using the Einstein–Stokes relation and the viscosity of water at 25 °C.

To investigate the interaction of hCT with EGCG, we added aliquots of the EGCG stock solution to prepare samples with different hCT:EGCG molar ratios. For these samples, 1D ¹H NMR, 2D NOESY, 2D TOCSY and ¹H/¹⁵N band-selective optimized flip-angle short transient (SOFAST) HMQC spectra were recorded in the absence and presence of EGCG. For experiments involving EGCG, NOESY spectra were obtained using 512 t_1 experiments, 300 ms of mixing time and 8 scans, while TOCSY spectra were obtained using 256 t_1 experiments, 80 ms of mixing time and 4 scans. The SOFAST HMQC spectra were obtained using 128 t_1 experiments and 256 scans.

Structure calculations

Structures were calculated from manually and automatically assigned NOEs in 2D NOESY spectra with a 200-ms mixing time using CYANA version 2.0.^{66,67} The normalized cross peak intensities were qualitatively assigned as strong, medium or weak to assign upper inter-proton distance restraints of 2.7 Å, 3.3 Å and 5.0 Å, respectively, for both 0.3- mM and 1- mM hCT samples.⁶⁸ An additional 0.5 Å was added to the upper bound for proton pairs involving methyl groups. For the 1.0- mM hCT sample, a total of 297 inter-proton distance restraints were derived from the NOESY data, including 175 sequential restraints ($i-j=1$), 19 medium-range restraints ($2 \leq i-j \leq 5$) and 2 long-range restraints [$(i-j>5)$ residues]. The combination of chemical shift restraints from TALOS and the NOE patterns were used to calculate 23 dihedral angle restraints for the 1- mM sample.⁶⁹ For the 0.3- mM hCT sample, long-range or medium-range NOEs were not observed except for 65 intraresidue and 66 sequential NOEs.

A total of 100 conformers for each sample were initially generated by CYANA based on the dihedral angle and NOE restraints obtained, and the bundles of 20 conformers with the lowest target function were used to represent the three-dimensional NMR structures. Hydrogen bond constraints were not included during the entire structure calculation. The

disulfide bridges between C1 and C7 were fixed in all structure calculations by constraining the S–S distance to an upper limit of 2.1 Å.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations used

1D	one-dimensional
2D	two-dimensional
hCT	human calcitonin
EGCG	epigallocatechin 3-gallate
H/D	hydrogen/deuterium
TOCSY	total correlated spectroscopy
NOE	nuclear Overhauser enhancement
NOESY	nuclear Overhauser enhancement spectroscopy
HMQC	heteronuclear multiple quantum coherence
SOFAST	band-selective optimized flip-angle short transient
PFG	pulsed-field gradient
DSS	4,4-dimethyl-4-silapentane-1-sulfonic acid

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Fig. 1.
Amino acid sequence of hCT.

Fig. 2. hCT forms oligomers at 1 mM concentration but not at 0.3 mM. (a) 1D ^1H NMR spectra of hCT at (a) 0.3 mM and (b) 1 mM concentrations showing the characteristic amyloid oligomer peak at -0.1 ppm in the 1- mM sample and its absence in the 0.3- mM sample. The peak at 0 ppm is from the internal chemical shift reference used (DSS).

Fig. 3.

hCT is primarily monomeric at both 0.3 and 1 mM concentrations. The decay of normalized STE intensity obtained from STE PFG ^1H NMR spectra of 0.3 mM (red circles) and 1 mM (blue squares) hCT samples; the intensity was calculated from the integrated volume of the peaks between 8.6 and 7.4 ppm. The similarity of the decays indicates that the peaks within this region correspond to largely unfolded monomeric species of hCT with a hydrodynamic radius of 1.57 nm.

Fig. 4. $\text{H}^{\alpha}\text{--}\text{H}^{\text{N}}$ region of the 2D NOESY spectrum of 1 mM hCT. The spectrum was recorded from 1 mM hCT sample in 2 mM sodium phosphate buffer, 7% D_2O and 50 mM NaCl (pH 2.9). Only the NOEs corresponding to cross peaks of amide protons and α protons are labeled.

Fig. 5. NOE connectivity plots for the 0.3- mM (a) and 1- mM (b) hCT samples. The strengths of sequential NOEs are indicated by the height of the bars.

Fig. 6.

Superimposed normalized NOESY spectra of hCT at. Superimposed normalized NOESY spectra of hCT at 0.3 and 1 mM concentrations. The $H^{\text{side chain}}_H$ - H^N regions of NOESY spectra at peptide concentration of 0.3 mM (blue) and 1 mM (magenta) are superimposed. The 1- mM spectrum was normalized by scaling the contour level down to that of the 0.3- mM sample, using the diagonal peak intensity as a reference. Additional NOEs were observed in the 1- mM sample, including intraresidue $H^{\text{side chain}}_H$ - H^N NOEs and interresidue ($i-i+1$) $H^{\text{side chain}}_H$ - H^N NOEs. Samples were prepared in 2 mM sodium phosphate buffer, 7% D_2O and 50 mM NaCl (pH 2.9).

Fig. 7. Partially folded structures of hCT. (a) A superimposition of five energy-minimized structures of hCT at the concentration of 0.3 mM. (b) A superimposition of 20 energy-minimized structures of hCT at the concentration of 1 mM. The peptide chains are labeled with gradient colors from blue to red throughout the sequence from N-terminus to C-terminus. (c) A cartoon plot showing the medium- and long-range NOEs (broken lines) that stabilize the formation of the hairpin structure at 1 mM concentration. Red broken lines indicate a hydrogen bond. (d) Positions and orientations of three aromatic side chains from five randomly chosen energy-minimized structures of the 1- mM sample of hCT.

Fig. 8.

Electron microscopy image shows the inhibition of hCT fibrillation by EGCG. Electron microscopy image shows the inhibition of hCT fibrillation by EGCG. We incubated 0.3 mM samples of hCT for 36 h in the absence (a) and presence (b) of 3 molar equivalents of EGCG at pH 7.4, 298 K.

Fig. 9.

EGCG inhibits oligomer formation of hCT by NMR. Time course of the amide region of 1D ^1H NMR spectra of hCT in H/D exchange experiments in the absence (a) and presence (b) of 2 molar equivalents of EGCG. (c) 1D ^1H NMR spectra of hCT in the presence (a) and absence (b) of 2 molar equivalents of EGCG before deuterium exchange. Samples were prepared in 2 mM sodium phosphate buffer, 7% D_2O and 50 mM NaCl (pH 2.9).

Fig. 10.

NMR analysis of EGCG binding to hCT. NMR spectra of hCT were recorded with and without 2 molar equivalents of EGCG. (a) 2D $^1\text{H}/^{15}\text{N}$ SOFAST HMQC spectra of hCT in the absence (blue) and presence (red) of EGCG. (b) Chemical shift difference of amide protons and α protons of hCT induced by the addition of EGCG. $\Delta\delta$ is calculated by subtracting the chemical shift values of protons of hCT in the presence of EGCG from those in the absence of EGCG. $\Delta\delta_{(\text{NH})}$ was calculated from 2D $^1\text{H}/^{15}\text{N}$ SOFAST HMQC spectra. $\Delta\delta_{(\alpha\text{H})}$ was calculated from 2D $^1\text{H}/^1\text{H}$ NOESY spectra. (c) Aromatic region of NOESY spectra of hCT in the absence (blue) and presence (red) of EGCG. Samples were prepared in 2 mM sodium phosphate buffer, 7% D_2O and 50 mM NaCl (pH 2.9).

Table 1

Structure statistics for the 20 best structures of hCT after energy minimization and structural annealing

	1 mM	0.3 mM
<i>Restraints for structure calculation</i>		
Total restraints	314	158
Total NOE restraints	291	131
Intraresidue	95	65
Sequential	175	66
Medium range	19	0
Long range	2	0
Dihedral angle restraints	23	27
NOE violations (number >0.2 Å)	0	0
Dihedral violations (number >3)	0	0
RMS deviation (Å) (residues 9–21)		
Backbone	0.97 ± 0.40	2.72 ± 0.52
Heavy atoms	1.69 ± 0.28	4.07 ± 0.43
Ramachandran data (%)		
Residues in the most favored regions	75.4	50.4
Residues in the additionally allowed regions	24.6	49.6
Residues in disallowed regions	0.0	0

Table 2

Selective medium- and long-range NOEs observed from the 2D NOESY spectrum of 1 mM hCT in sodium phosphate and 50 mM NaCl (pH 2.9) at 298 K

From residue	To residue
T13-QG2	F19-QD
T13-HA	F19-QD
T13-HA	N17-QB
T13-QG2	N17-QB
T13-QG2	N17-QD
Q14-HN	N17-HD21
Q14-HN	N17-HD22
Q14-HN	N17-QB
Q14-HN	N17-QD2