The Structure and Mechanism of Formation of Human Calcitonin Fibrils

Tudor Arvinte††, Amelia Cudd††, and Alex F. Drake†

From †Ciba-Geigy Pharmaceuticals, Horsham RH12 4AB, United Kingdom and the ††Department of Chemistry, Birkbeck College, University of London, London WC1H 0AJ, United Kingdom

Turbidity measurements of the kinetics of human calcitonin (hCT) fibrillation showed a linear dependence of the logarithm of fibrillation time (the time the sample is not fibrillated) and the logarithm of hCT concentration. This ln/ln plot linearity and electron microscope observations of fibrils indicate that the fibrillation process can be explained by the double nucleation mechanism that was proposed for the gelation of sickle cell hemoglobin (Ferrone, F. A., Hofrichter, J., Sunshine, H. R., and Eaton, W. A. (1980) Biophys. J. 32, 361–380). Circular dichroism, fluorescence, and infrared spectroscopy studies of fibrils showed that hCT molecules have α-helical and β-sheet secondary structure components. A model for the structure of hCT molecules in fibrils is proposed.

Calcitonin (CT) is a peptide hormone which, due to its regulatory function in calcium-phosphorus metabolism (Copp et al., 1962; Kumar et al., 1963; Austin and Heath, 1981), is used as a drug for various bone disorders such as Paget’s disease and osteoporosis. Salmon, human, pig, and eel calcitonins are currently used therapeutically. Human calcitonin (hCT) has a tendency to associate and precipitate in solution. Electron microscope observations of the resulting viscous and gelatinous hCT solution showed that it consists of fibrils of 80 Å in diameter that often associate with one another (Sieber et al., 1970). The fibrillation process was shown to be reversible; heating of fibrillated hCT solutions in 50% acetic acid/water converts the fibrils to soluble hCT monomers (Sieber et al., 1970).

A wide range of proteins and peptides form fibrils in aqueous solutions, e.g. insulin (Waugh, 1944; Waugh et al., 1952), glucagon (Staub et al., 1955), serum albumin (Jaggi and Staub, 1950), fibrin (Erlich et al., 1952), sickle cell hemoglobin (Dykes et al., 1978), collagen (Randall et al., 1955), glutamine synthetase (Frey et al., 1975), microtubule solutions (Hitt et al., 1990), transthyretin (Gustavsson et al., 1991), and islet amyloid polypeptide (Westermak et al., 1990).

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† Present address: The William Harvey Research Institute, St. Bartholomew’s Hospital Medical College, London EC1M 6BQ, United Kingdom.
‡ To whom correspondence should be addressed: Ciba-Geigy Pharmaceuticals, Wilmington Rd., Horsham RH12 4AB, United Kingdom. Tel.: 44-403-272872 or 44-403-323711; Fax: 44-403-323253.

1 The abbreviations used are: CT, calcitonin; hCT, human calcitonin; CD, circular dichroism; FITC, fluorescein isothiocyanate isomer I; FTIR, Fourier transform infrared; PBS, phosphate-buffered saline.

Extracellular deposits of protein fibrillar structures associated with different diseases are referred to as amyloid (for a review, see Goffin (1989)). Amyloids are associated with type 2 diabetes (Clark et al., 1987), medullary thyroid carcinomas (Sletten et al., 1976), or neurological diseases such as Alzheimer’s disease (Crowther, 1991), postencephalitic Parkinsonism (Ishii and Nakamura, 1981), Guam disease (Hirano et al., 1968), progressive supranuclear palsy (Ghatak et al., 1980), or Hallervorden-Spatz disease (Eidelberg et al., 1987). In the majority of cases, little is known about the identity of the proteins/peptides that form the filaments. Sletten et al. (1976) have provided evidence that amyloid fibril proteins from medullary carcinoma of the thyroid have an amino acid composition similar to that of human calcitonin but of a larger size; they proposed that the amyloid fibril consists of procalcitonin. Studies of tumors of medullary carcinoma of the thyroid identified human calcitonin immunoreactivity within secretory granules of the tumor cells (DeLellis et al., 1978; Dämmrich et al., 1984) and also within the intra- or extracellular amyloid fibrils (Butler and Khan, 1986; Silver et al., 1988; Berger et al., 1988; Byard et al., 1990). It was suggested that calcitonin (and/or pro-calcitonin) may transform into amyloid before it is secreted into the interstitial space, within the secretory granule (Silver et al., 1988).

There are indications that hCT may be secreted in aggregated/fibrillated forms in nonpathological conditions. Thus, immunoreactive studies showed that high molecular mass forms of calcitonin are present in the circulation of healthy humans (Woloszczuk et al., 1986; Motté et al., 1986; Wimalawansa, 1990; Bucht et al., 1990) and that the monomeric form of hCT accounts for a fraction of the tct immunoreactive hCT in circulation (Wimalawansa, 1990).

Here, we report our studies of hCT fibrillation. Turbidity measurements of the kinetics of hCT fibrillation and electron microscope observations of fibrils indicate that the fibrillation process can be explained by the double nucleation mechanism that was proposed for the gelation of sickle cell hemoglobin (Ferrone et al., 1980, 1985; Samuel et al., 1990). Circular dichroism (CD), fluorescence, and infrared spectroscopy studies of fibrils showed that hCT molecules have α-helical and β-sheet secondary structure components. A model for the hCT conformation changes associated with fibrillation is proposed.

EXPERIMENTAL PROCEDURES

Materials—Synthetic human calcitonin was obtained from Ciba-Geigy Pharmaceuticals, Basel, Switzerland. Synthetic sCT was purchased from Novabiochem, Switzerland. Fluorescein isothiocyanate isomer I (FITC), SDS, and Tris were from Sigma, Poole, United Kingdom. All other chemicals were of analytical grade.

Peptide Concentration—Calcitonin concentration was determined from its weight concentration or from its ultraviolet absorption spectrum. Absorption coefficients at 275 nm of 15.51 and 15.15 cm−1 M−1 were used for hCT and sCT, respectively (Epand et al., 1985).
Calcitonin Labelling with FITC—Covalent binding of FITC to human calcitonin was performed according to Goldman (1968). 0.9 ml of hCT solution in water (0.5 mg/ml hCT) was mixed with 100 µl of sodium carbonate buffer (0.5 M carbonate buffer, pH 9.5, 0.145 M NaCl) containing 0.4 mg of FITC. This mixture was incubated in the dark at 21 °C for 1 h. The unbound FITC was then separated from hCT by filtration through Sephadex G-25 columns. The elution solution was 0.001% acetic acid. The fractions containing the fluorescently labelled hCT in 0.001% acetic acid were easily recognized under a fluorescent lamp. Calcitonin concentration in the samples was calculated from absorbance measurements at 275 nm after correcting for the absorbance of FITC at that wavelength (Goldman, 1968) and from calcitonin tyrosine fluorescence at 305 nm, excitation at 275 nm. The amount of FITC bound to hCT was calculated from the fluorescein absorption spectrum of labeled hCT at 496 nm using an absorption coefficient of 80,000 for fluorescein bound to proteins (Simpson, 1978; Pick and Karlish, 1980). An average labeling of 1 FITC molecule/1.7 molecules of hCT was obtained. When the above procedure was applied at a higher hCT concentration (5 mg/ml hCT instead of 0.5 mg/ml hCT), an average labeling of 1 FITC molecule/31 hCT molecules was obtained.

Absorption Measurements—Absorption spectra were recorded using a dual-beam Perkin-Elmer Lambda 5 spectrometer at 20 °C. Fibril formation in a calcitonin solution was monitored by measuring the changes in turbidity at 340 nm with time.

Fluorescence Measurements—Fluorescence emission spectra were measured with a Perkin-Elmer LS 5 spectrofluorimeter at 20 °C. The tyrosine fluorescence of hCT was excited at 270 nm, and the emission was monitored between 280 and 490 nm. FITC was excited at 470 nm, and the maximum fluorescence intensity was measured from emission spectra recorded between 450 and 600 nm.

Measurements of fluorescence polarization were made using filter polarizers in the excitation and emission beams. Fluorescence polarization degrees were calculated from the equation

\[ P = \left( \frac{I_{90} - G \times I_{45}}{I_{90} + G \times I_{45}} \right) \]

where G is a correction factor, \( I_{90} = I_{90}/I_{45} \) (Azumi and McGlynn, 1962). \( I_{90} \) is the maximum fluorescence intensity and was measured from emission spectra; the subscripts refer to the positions of polarizers in the excitation and emission beams relative to the vertical axis. A correction for light scatter was made for each fluorescence intensity by subtracting the value obtained for FITC-unlabeled sample, under identical conditions.

Circular Dichroism—CD spectra were obtained using a JASCO J600 spectropolarimeter equipped with a thermostatted cell holder. Estimates of peptide secondary structure were performed using the standard CONTIN program (190-240 nm) (Provencher and Glöckner, 1981). For the hCT fibrils investigation a Hellma spacer cell (210.003-QS) was used (Hellma, Westcliff-on-Sea, United Kingdom).

Infrared Spectroscopy—Fourier transform infrared (FTIR) spectra were obtained with a Perkin-Elmer 1750 infrared Fourier transform spectrometer at 20 °C. Typically, the spectra were averaged over 20 scans.

In all spectroscopic investigations, reference spectra were recorded under identical conditions with only the media in which the peptides

Fig. 1. Typical change in time of the absorption of an hCT solution due to fibrillation.

Fig. 2. Concentration dependence of the fibrillation process. hCT was solubilized in water. hCT water solutions were mixed with phosphate buffer to give a final solution of 5 mM phosphate buffer, pH 7.4, 145 mM NaCl (PBS).

Fig. 3. Temperature dependence of the fibrillation process. Samples of 10 mg/ml hCT (hCT stock solutions in water) in PBS were incubated at the indicated temperatures.
were dissolved in the cells; these spectra were subtracted from the corresponding peptide spectra.

**Electron Microscopy**—For negative staining, a drop of hCT suspension was placed on a Formvar-covered grid, and excess fluid was wicked off. Filtered ammonium molybdate, 0.7%, pH 5.38, was added and wicked after 30 s. Samples were freeze-fractured without modification, i.e., they were not concentrated and no cryoprotectants were used. Samples were quick frozen in a liquid nitrogen jet (Polaron), and double replicas were made at −150 °C in a Polaron E7500 freeze-fracture device. Platinum/carbon shadowing at 45° was followed by carbon coating at 90 °C from the sample surface. Replicas were removed by floating on water or a chloroform/methanol solution and cleaned with 5% bleach for 10 min. Replicas and grids from negative staining were viewed in a Philips CM 10 or Philips 300 electron microscope at 60 kV.

**RESULTS**

**Turbidity Measurement of hCT Fibrillation**—The kinetic measurements were based on the increase in turbidity at 340 nm from a clear hCT solution. Similar methods have been used for measuring the kinetics of fiber formation from collagen solutions (Bensusan and Hoyt, 1958) or for the assembly of neurotubes (Gaskin et al., 1974). Fig. 1 shows a typical change in time of the absorption of a hCT solution due to fibrillation in 5 mM phosphate buffer, pH 7.4, 145 mM NaCl. The following steps can be observed by eye (see Fig. 1, inset); (i) a time period during which no changes can be detected, referred to as lag time, delay time, or fibrillation time; (ii) the start of fibrillation, which is marked by the occurrence of small particulate aggregates; and (iii) the fibrillation, a period during which the solution becomes increasingly turbid and viscous. The fibrillation time (t) was defined as the time corresponding to the intersection with the time axis of the linear increase in turbidity; see Fig. 1. The fibrillation process is dependent on hCT concentration (Fig. 2). The end fibrillated state of the hCT solutions in Fig. 2 has a different appearance for different initial hCT concentrations, (i) for

| [CT]  | Human calcitonin | Salmon calcitonin |
|-------|------------------|------------------|
| mg/ml | min              |                  |
| 20    | 1                | ~21 days         |
| 10    | 3                | ~70 days         |
| 7.5   | 4                | ~100 days        |
| 5     | 5                | ~7 months        |
| 1     | 21               | >8 months        |

**Fig. 4. Negative staining electron micrographs of the initiation of fibrillation of hCT in water.** Samples from a 100 mg/ml hCT solution were taken for staining 7 min after hCT was dissolved. After 7 min, the solution of 100 mg/ml hCT was fluid and slightly turbid, indicating the onset of fibrillation.
hCT concentrations below and around 1 mg/ml hCT, the fibrillated equilibrium state consists of a clear solution containing punctate aggregates that precipitate; (ii) for increased hCT concentrations, 2–15 mg/ml, the end fibrillated state is a turbid viscous solution; (iii) for concentrations above 15 mg/ml hCT, the equilibrium state is a hard, turbid gel. As shown in the inset in Fig. 2, the fibrillation time-concentration dependence could be fitted with a straight line in a ln/ln representation. This linearity was constantly obtained in different experimental conditions (data not shown). Fibrillation was temperature-dependent (Fig. 3). From Arrhenius plots of the data in Fig. 3 (not shown), an apparent activation enthalpy for the fibril formation of 20 kcal/mol was obtained. This value is similar to the value of 21 kcal/mol measured for the apparent reaction enthalpy of assembly for the chain-propagating step of neurotubules (Gaskin et al., 1974). Human calcitonin fibrillates much faster than salmon calcitonin (Table 1).

**Electron Microscope Characterization of hCT Fibrils**—The initiation of fibrillation of human calcitonin in water is shown by negative staining electron microscopy (Fig. 4). Samples from a 100 mg/ml hCT solution were taken for staining 7 min after hCT was dissolved. After 7 min, the solution of 100 mg/ml hCT is fluid and slightly turbid, indicating the onset of fibrillation (the end fibrillation state for the sample is a turbid hard gel of hCT fibrils). Fig. 4A shows immature fibrils of various thickness and consistency and at least one case of fibrils emerging from areas of coalesced hCT aggregates (arrow). Immature fibrils ranged in diameter from about 6 to 40 nm. Another part of the same grid (i.e. the same sample of the same specimen, prepared at the same time) showed more fibrils with stellate areas of concentration (Fig. 4B, arrow). These fibrils were more uniform in diameter; the smallest observed diameter was about 18 nm.

Electron micrographs obtained from fully fibrillated samples of hCT in water are shown in Fig. 5 (negative staining) and Fig. 6 (freeze-fracture). The smallest fibrils observed by negative staining had diameters of about 2 nm (Fig. 4B). In many cases, these were organized into larger fibrils of 8–17 nm in diameter. Bundles of hCT fibrils were often observed (Fig. 5, arrows). It is not evident from negative staining that the smaller fibrils are also helically organized into larger fibrils, but this is observed in the freeze-fracture images (Fig. 6, arrows). The diameter of the smallest unit fibrils observed by freeze-fracture was 8 nm.

**CD Study of hCT Fibrillation**—In aqueous solutions, hCT has a circular dichroism spectrum indicating an unordered secondary structure (Epand et al., 1983; Arvinte and Drake, 1992). The CD spectrum of hCT fibrils is shown in Fig. 7, curve 2. The spectrum of hCT fibrils in Fig. 7 was obtained from a gel of hCT fibrils that was formed in a vial (solution volume 250 μl, vial of 1 cm diameter); we will refer to hCT fibrils that were formed under large volume conditions as mature fibrils. A small piece from this hCT fibrillated gel (mature fibrils) was pressed between the silica plates of the 0.01-mm CD cell. The CD spectrum of the mature hCT fibrils

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**Fig. 5.** Negative stain of fully fibrillated samples of hCT in water. Samples of 100 mg/ml hCT in water were incubated for 24 h at room temperature.
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FIG. 6. Freeze-fracture of hCT fibrils. The samples, 100 mg/ml hCT, were incubated for 24 h at room temperature prior to the freeze-fracture.

(Fig. 7) is very similar to the CD spectrum of hCT in methanol (Fig. 7, curve 1) indicating the presence of 25% α-helix content (CONTIN analysis, see “Experimental Procedures”). The CD spectral changes monitored during the transition from a nonfibrillated to a fibrillated hCT solution are shown in Fig. 8. Fibrillation times in the narrow path CD cuvette were found to be much longer than in a 1-cm diameter glass vial (200–250-μl volume samples); for this reason, the fibrillation in CD cuvettes was induced by warming the cuvette at 40 °C for 1 min. Comparison with the CD spectrum of hCT in methanol (Fig. 9) shows that the equilibrium state of hCT solutions fibrillated in the CD cuvette is different from the CD spectrum of hCT fibrillated outside the cuvette (Fig. 7). We will refer to the hCT fibrils formed inside the CD cuvette as hindered fibrils due to the restricted geometrical conditions (0.01-mm path length of the CD cuvette), which suppress the formation of mature hCT fibrils. Visual observation of the hindered fibrils in the cuvette showed the coexistence of fibrillar structures and granular, punctate aggregates. Secondary structure estimates from the CD spectra (Figs. 7 and 9) showed that in the equilibrium state, hindered fibrils have an α-helical content of 17% (CONTIN analysis), which is less than the value of 25% for the mature fibrils.

Infrared Spectroscopy Study of hCT Fibrillation—Fig. 10 illustrates the FTIR spectral changes associated with hCT fibrillation in 5 mM phosphate buffer, pH 7.4, 145 mM NaCl (PBS) buffer made in D₂O. These changes correspond to the formation of hindered hCT fibrils since this IR experiment requires a narrow cuvette (0.01-mm path length). Granular, punctate aggregates were observed in the IR cuvette at the end of the fibrillation experiment (Fig. 10). The transition from a clear hCT solution to a fibrillated hCT solution strongly influences the amide I region; there is a reduction in the band at 1650 cm⁻¹ and the appearance of a strong absorption at 1620 cm⁻¹ (Fig. 10). Amide I absorption bands in the region of 1630 cm⁻¹ are generally associated with β-sheets (i.e. Dong et al., 1990; Dousseau and Pézolet, 1990). The FTIR spectrum of a fibrillated hCT solution is different from the FTIR spectrum of hCT in methanol, a condition in which hCT adopts a full α-helical structure (Arvinte and Drake, 1992).

Fluorescence Study of hCT Fibrils—The fluorescence properties of the single tyrosine in the sequence of hCT can be used to monitor changes in hCT secondary structure (Arvinte and Drake, 1992). hCT fibrillation induced a strong increase in Tyre fluorescence and polarization degree; it increased from the uniformly low value of \( p = 0.090 \pm 0.01 \) for a nonfibrillated hCT solution to \( p = 0.480 \pm 0.02 \) for a completely fibrillated hCT solution (mature fibrils). The p-value of 0.480 indicates that in the fibrillated state, the Tyre is in a rigid environment (the maximal possible polarization value for a rigid system is 0.500 in the case in which absorbing and emitting transition dipoles of the chromophore are parallel (i.e. Cantor and Schimmel, 1980)).

Further information on the hCT fibrils was obtained using FITC-labeled hCT. Lysine residues and the terminal amino
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Fig. 7. hCT fibrils contain α-helical secondary structure component. Curve 1, the CD spectrum of hCT in methanol (48% α-helix; Arvinte and Drake (1992)). Curve 2, the CD spectrum of hCT fibrils obtained from a gel of hCT fibrils that was formed in a vial (40 mg/ml hCT in water; solution volume, 250 µl; vial, 1-cm diameter; 48 h at room temperature). A small piece from this hCT fibrillated gel was pressed between the silica plates of the 0.01-mm CD cuvette.

Fig. 8. The CD spectral changes monitored during the transition from a nonfibrillated to a fibrillated hCT solution (30 mg/ml hCT in PBS). Fibrillation was induced by warming the cuvette at 40 °C for 1 min. No changes in CD were obtained in the first 10 min. The curves correspond to CD spectra measured 0, 10, 20, 30, 45, 60, and 1000 min after mixing an hCT water solution with phosphate buffer (5 mM phosphate buffer, pH 7.4, 145 mM NaCl). Eye observation of the fibrils at equilibrium showed the coexistence of fibrillar structures and granular, punctate aggregates.

Fig. 9. The equilibrium state of hCT solutions fibrillated in the CD cuvette, curve 2 (corresponding to the equilibrium in Fig. 8), is different from the CD spectrum of hCT in methanol, curve 1.

Fig. 10. FTIR spectral changes associated with hCT fibrillation in PBS buffer made in D$_2$O, 20 mg/ml hCT.

Table II

| hCT solution | FITC fluorescence ($F_{nm}/F_0$) | TyP fluorescence ($F_{nm}/F_0$) |
|--------------|---------------------------------|--------------------------------|
| Nonfibrillated | 2.9 | 21.0 |
| Fibrillated | 2.3 | 1.2 |

Fluorescence intensity measurements of FITC-labeled hCT in the presence and absence of SDS

FITC fluorescence intensity of FITC-hCT samples in the absence (F$_0$) and in the presence of 0.5% SDS (F$_{nm}$) were measured from emission spectra (exitation, 470 nm). The nonfibrillated sample had a concentration of 0.13 mg/ml hCT; the fibrillated sample had a concentration of 2.7 mg/ml hCT. The fibrillated sample consisted of fibrillar structures and granular aggregates that formed after 6 weeks. Both samples were in 0.001% acetic acid.

Fluorescence intensity measurements of FITC-labeled hCT in the presence and absence of SDS

Fluorescence intensity measurements of FITC-labeled hCT in the presence and absence of SDS are shown in Table II. Addition of SDS over a hCT nonfibrillated solution results in an increase in the TyP$^{12}$ and the FITC fluorescence intensities. However, addition of SDS over a fibrillated hCT solution results only in an increase in the FITC fluorescence intensity, with practically no effects on the TyP$^{12}$ fluorescence intensity (Table II). These fluorescence changes (Table II) indicate that in the fibrils, hCT molecules
are arranged so that Tyr^{12} is in a hydrophobic environment (not accessible to SDS) and that the fluorescein bound to hCT is in a hydrophilic environment. Since Lys is a major binding site of FITC to proteins (Goldman, 1968), our experiments indicate that in fibrils, hCT molecules have Lys^{18} in a hydrophobic environment (accessible to SDS). This arrangement, Tyr^{12} in a hydrophobic and Lys^{18} in a hydrophilic environment, is predicted by the amphipathic α-helix wheel representation of residues 8–22 of hCT (Epand et al., 1983). Thus, the fluorescence data show that the spatial location of Tyr^{12} and Lys^{18} is in agreement with the proposed amphipathic α-helical structure of hCT. This supports the CD results, which showed that the mature hCT fibrils contain an α-helical structural component.

DISCUSSION

The kinetics of calcitonin fibrillation showed a linear dependence of the logarithm of the fibrillation time versus the logarithm of concentration (Fig. 2). Similar linear ln/ln dependence was also observed for sickle cell hemoglobin (Hofrichter et al., 1974, 1976), and a model explaining the linearity was proposed (Hofrichter et al., 1974). The observed linearity of the ln/ln plot in the case of hCT (Fig. 2) suggests that the fibrillation mechanisms of hCT and sickle cell hemoglobin are similar. To explain the fibrillation of sickle cell hemoglobin, a double nucleation mechanism was developed (Ferrone et al., 1980, 1985; Samuel et al., 1990). In the double nucleation mechanism, fibrillation starts with a homogeneous nucleation step in which spherical aggregates and first fibrils are formed from protein monomers. Electrostatic interactions between calcitonin monomers are likely to play an important role in the initial aggregation step. In this context, the different pH values of human and salmon calcitonin (hCT has a pK of 8.7; sCT has a pK of 9.4) are similar. To explain the fibrillation of sickle cell hemoglobin, a double nucleation model proposed for sickle cell hemoglobin fibrillation was extended to hCT. The possibility that the double nucleation model has a general validity in explaining the fibrillation mechanisms of hCT and sickle cell hemoglobin fibrils (Arvinte and Ryman, 1990). The human calcitonin fibrillation starts with a homogeneous nucleation step in which spherical aggregates and first fibrils are formed from protein monomers. The understanding of the fibrillation process and of the hCT folding mechanism will contribute to the improvement of the recently developed long time stable aqueous therapeutic formulations of hCT (Arvinte and Ryman, 1992) and the potential formulations based upon hCT fibrils (Arvinte et al., 1992).

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