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Identification of a novel 'aggregation-prone'/'amyloidogenic determinant' peptide in the sequence of the highly amyloidogenic human calcitonin

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ABSTRACT

Calcitonin is a 32-residue polypeptide hormone, which takes part in calcium metabolism in bones. It may form amyloid fibrils. Amyloid fibrils are related with serious diseases known as amyloidoses. The amyloid form of calcitonin takes part in medullary thyroid carcinoma. A novel hexapeptide (^{6}TCMLGT^{11}) of human calcitonin was predicted as a possible 'aggregation-prone' peptide, which may play a role in amyloid formation. We investigated experimentally the ability of an analog of this hexapeptide (cysteine replaced by alanine, TAMLGT) to form amyloid fibrils utilizing TEM, X-ray fiber diffraction, ATR FT-IR spectroscopy, and polarized light microscopy. This peptide self-assembles into amyloid-like fibrils and fibrillogenesis is mediated via nuclei of liquid crystalline nature, known as spherulites.

Structured summary of protein interactions:
TAMLGT peptide and **TAMLGT peptide**bind by x-ray fiber diffraction (View interaction)
TAMLGT peptide and **TAMLGT peptide**bind by infrared spectroscopy (View interaction)
TAMLGT peptide and **TAMLGT peptide**bind by electron microscopy (View interaction)

1. Introduction

A great number of proteins and peptides with different functions and various structures are grouped together because of their ability to form ordered fibrillar aggregates, known as amyloid fibrils. These fibrils share distinct, common features: they bind Congo Red and display a characteristic yellow-green birefringence under polarized light [1], they are seen, utilizing electron microscopy, as uniform, straight or slightly curved, unbranched and of indefinite length fibrils, approximately 100 Å in diameter [2], and, finally they produce cross-β X-ray fiber diffraction patterns [3–5]. Amyloid fibrils are related to diseases, such as Alzheimer's disease, atherosclerosis, cerebral amyloid angiopathy (Icelandic type), type II diabetes, medullary thyroid carcinoma, which are, collectively, called amyloidoses [6]. However, there are cases where certain proteins or peptides with an amyloid-forming potency play important functional roles in some organisms, including insects [4], bacteria [7] and even humans [8].

Calcitonin, a 32-amino acid polypeptide hormone [9] (Fig. 1), is produced in mammals by the C-cells of the thyroid gland and in lower vertebrates by the ultimobranchial body [10]. This hormone (hereinafter called also hCT from human CalciTonin) is involved in calcium-phosphorus metabolism [10]. Therefore, it is used as a drug to treat various bone disorders like postmenopausal osteroposis [11], Paget's disease [12] and also for short-term management of severe symptomatic hypercalcaemia [13]. It has been found that deposition of full-length calcitonin amyloid fibrils is associated with medullary thyroid carcinoma [14,15]. More specifically it has been proved that full-length calcitonin hormone and not an alternatively processed prohormone of calcitonin, forms amyloid in medullary thyroid carcinoma [16]. It has also been found that human calcitonin (hCT) may aggregate in vitro under specific incubation conditions. Many studies have been done about the mechanisms and kinetics of calcitonin fibrillation [17,18].

It has been shown that five residues corresponding to positions 15–19 of hCT (DFNKF) play an active role in oligomerisation and fibril formation by hCT in vitro [19], and Lys-18 and Phe-19 have been identified as key residues in both the bioactivity and self-assembly of hCT [20]. Attempts were also made to convert the highly amyloidogenic hCT into a powerful fibril inhibitor by three-dimensional structure homology with a non-amyloidogenic

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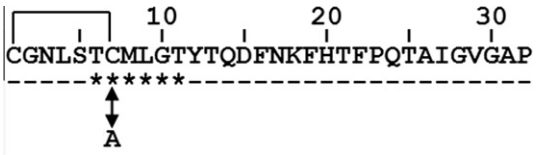

Fig. 1. A schematic representation of the aminoacid sequence of mature human calcitonin (hCT), which consists of 32 amino acid residues, is shown. There is a disulfide bridge between Cys1 and Cys7 in mature calcitonin, essential for its biological action, which is depicted by straight lines connecting the two residues, above the sequence. A predicted 'amyloidogenic determinant'/'aggregation-prone' peptide (^{6}TCMLGT^{11}) by our algorithm AMYLPRED [23] is marked by "$*$" (asterisks) below the sequence. An analog of this peptide (^{6}TAMLGT^{11}), by replacing cysteine (C) with alanine (A) (double arrow), was synthesized and studied in this work. This peptide was also predicted to be 'amyloidogenic determinant'/'aggregation-prone' by AMYLPRED [23].

analog [21], and, also to rationally design an aggregation-resistant bioactive calcitonin [22].

AMYLPRED is a consensus prediction algorithm for amyloid fibril favoring regions, the so-called amyloidogenic determinants/'aggregation-prone' stretches, which was produced in our lab and is freely available for academic users at http://biophysics.biol.uoa.gr/AMYLPRED [23]. Testing the hCT sequence by AMYLPRED, the hexapeptide TCMLGT (residues 6–11 of the protein), was predicted as a possible amyloidogenic determinant/'aggregation-prone' sequence. We synthesized an analog of this peptide by replacing cysteine with alanine in order to prevent the formation of intermolecular disulfide bonds between cysteines, at approximately the neutral pH we wanted to perform the experiments. We should note that, the 'mutant' peptide TAMLGT was predicted by AMYLPRED as an 'aggregation-prone' peptide as well, in the 'mutated' calcitonin sequence.

In this work, the hexapeptide TAMLGT was tested experimentally for fulfilling the criteria stated above, to see whether it is capable of forming amyloid fibrils or not. It will be shown below that the tests were positive and that this hexapeptide self-assembles to form amyloid fibrils, via nuclei of liquid crystalline nature, known as spherulites [24]. The fact that nuclei (spherulites) with a liquid crystalline texture act as pre-fibrillar intermediates in amyloid fibril formation, has been observed in several other peptide or protein systems forming amyloid fibrils [24].

2. Materials and methods

2.1. Peptide synthesis

The TAMLGT peptide was synthesized by GeneCust Europe, Luxembourg (purity >98%, free N- and C-terminals).

2.2. Formation of amyloid-like fibrils

The synthesized TAMLGT peptide was dissolved in doubly distilled water (pH 5.5), at a concentration of 15 mg ml^{-1}. Mature amyloid-like fibrils were formed after 1–2 weeks incubation at ambient (room) temperatures, forming a fibril-containing gel. The fibrils were judged to be mature, observing preparations both for shorter and longer periods than 1–2 weeks. Oriented fibers, suitable for X-ray diffraction, were obtained from solutions of the peptide, containing mature amyloid-like fibrils as described below.

2.3. X-ray diffraction

A droplet (~10 μl) of mature fibril suspension was placed between two siliconized glass rods, spaced ~1.5 mm apart and mounted horizontally on a glass substrate, as collinearly as possible. The droplet was allowed to dry slowly at ambient temperature and humidity for 1 h to form an oriented fiber suitable for X-ray diffraction. X-ray patterns were obtained immediately from these fibers since it was found that fibers were drying and destroyed, after ~2 h under these conditions. X-ray diffraction patterns were recorded on a Mar Research 345 mm image plate, utilizing Cu Kα radiation (λ = 1.5418 Å), obtained from a Rigaku MicroMax-007 HF, microfocus rotating anode generator (with Osmic Rigaku VariMax™ HF optics), operating at 40 kV, 20 mA. The specimen-to-film distance was set at 150 mm and the exposure time was 30 min. No additional low angle reflections were observed at longer specimen-to-film distances, up to 300 mm. The X-ray patterns, initially viewed using the program MarView (MAR Research, Hamburg, Germany), were displayed and measured with the aid of the program IPDISP of the CCP4 package [25].

2.4. Negative staining

For negative staining, the TAMLGT peptide fibril suspensions were applied to glow-discharged 400 mesh carbon coated copper grids for 60 s. The grids were (occasionally) flash-washed with ~150 μl of distilled water and stained with a drop of 1% (w/v) aqueous uranyl acetate for 45 s. Excess stain was removed by blotting with a filter paper and the grids were air-dried. Pictures were acquired in a Philips CM120 BioTWIN electron microscope (FEI, Eindoven, The Netherlands) operating at 100 kV. Digital acquisitions were made with a bottom-mounted Keen View 1K CCD camera (Soft Imaging System, Muenster, Germany).

2.5. Congo Red staining and polarized light microscopy

TAMLGT fibril suspensions were applied to glass slides and stained with a 10 mM Congo Red (Sigma) solution in phosphate-buffered saline (pH 7.4) for approximately 2 h. They were then washed several times with 90% ethanol and left to dry. The samples were observed respectively, both under bright field illumination and between crossed polars using a Leica MZ75 polarizing stereomicroscope equipped with a JVC GC-X3E camera.

2.6. Attenuated total reflectance Fourier-transform infrared (ATR FT-IR) spectroscopy and post-run computations of the spectra

Ten microliter drops of the TAMLGT peptide suspension (Section 2.2) were cast on flat stainless-steel plates, coated with an ultra thin hydrophobic layer (SpectRIM, Tienta Sciences, Inc. Indianapolis, USA) and left to air-dry slowly at ambient conditions to form thin films. IR spectra were obtained from these films, at a resolution of 4 cm^{-1}, utilizing an IR microscope (IRScope II, Bruker Optik GmbH, Ettlingen, Germany), equipped with a Ge ATR objective lens (20×) and attached to a FT-IR spectrometer (Equinox 55, Bruker Optik GmbH, Ettlingen, Germany). Ten 32-scan spectra were collected from each sample and averaged to improve the S/N ratio. Internal reflection spectroscopy has several advantages compared to the more common KBr dispersion technique [26]. The choice of ATR was dictated by the need to exclude any possible spectroscopic and chemical interactions between the sample and the dispersing medium. Having a penetration depth of less than 1 μm (1000 cm^{-1}, Ge), ATR is free of saturation effects, which may be present in the transmission spectra of thicker samples. The spectra were corrected for the effect of wavelength on the penetration depth (p.d. $\propto \lambda$). The corresponding effect of the (frequency-dependent) refractive index (n) of the samples was not taken into account due to the lack of relevant data.

The infrared ATR band maxima were determined from the minima in the second derivative of the corresponding spectra. Derivatives were computed analytically using routines of the Bruker