Elucidating the role of disulfide bond on amyloid formation and fibril reversibility of somatostatin-14: Relevance to its storage and secretion*

Arunagiri Anoop1€, Srivastav Ranganathan1€, Bhagwan Das Dhaked1, Narendra Nath Jha1, Supriya Pratihar2, Saikat Ghosh1, Shruti Sahay1, Santosh Kumar3, Subhadeep Das14, Mamata Kombrabail2, Kumud Agarwal1, Reeba S. Jacob1, Praful Singru3, Prasenjit Bhaumik1, Ranjith Padinheateri1, Ashutosh Kumar1 and Samir K. Maji1

1Department of Biosciences and Bioengineering, Indian Institute of Technology Bombay, Mumbai, India
2Department of Chemical Sciences, Tata Institute of Fundamental Research, Mumbai, India
3School of Biological Sciences, National Institute of Science Education and Research, Bhubaneswar, India
4IITB-Monash Research Academy, Indian Institute of Technology Bombay, Mumbai, India

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To whom correspondence should be addressed: Samir K. Maji, Department of Biosciences and Bioengineering, IIT Bombay, Mumbai 400 076, India, Tel: + (91-22) 2576-7774, Fax: + (91-22) 2572 3480, Email: samirmaji@iitb.ac.in, Ashutosh Kumar, Department of Biosciences and Bioengineering, IIT Bombay, Mumbai 400 076, India, Tel: + (91-22) 2576-7762, Email: ashutoshk@iitb.ac.in

Keywords: Peptide hormones; somatostatin-14; disulfide; peptide conformation; amyloid; aggregation; storage; secretion

Background: Peptide/protein hormones are stored as amyloids within endocrine secretory granules

Result: Disulfide bond cleavage enhances conformational dynamics and aggregation kinetics in somatostatin-14, resulting in amyloid fibrils with increased resistance to denaturing conditions and decreased reversibility

Conclusion: Disulfide bond could be a key modulating factor in somatostatin-14 amyloid formation associated with secretory granule biogenesis

Significance: Defective disulfide bonding might cause dysregulation of hormone storage/secretion

ABSTRACT

The storage of protein/peptide hormones within sub-cellular compartments and subsequent release are crucial for their native function, and hence these processes are intricately regulated in mammalian systems. Several peptide hormones were recently suggested to be stored as amyloids within endocrine secretory granules. This leads to an apparent paradox where storage requires formation of aggregates, while their function requires supply of non-aggregated peptides on demand. The precise mechanism behind amyloid formation by these hormones and their subsequent release remain an open question. To address this, we examined aggregation and fibril reversibility of a cyclic peptide hormone somatostatin-14 (SST) using various techniques. After proving that SST gets stored as amyloid in vivo, we investigated the role of...
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native structure in modulating its conformational dynamics and self-association by disrupting the disulfide bridge (Cys\(^3\)-Cys\(^{14}\)) in SST. Using 2D-NMR, we resolved the initial structure of somatostatin-14 leading to aggregation and further probed its conformational dynamics in silico. The perturbation in native structure (S-S cleavage) led to a significant increase in conformational flexibility and resulted in rapid amyloid formation. The fibrils formed by disulfide-reduced ‘non-cyclic’ somatostatin (ncSST) possess greater resistance to denaturing conditions with decreased monomer releasing potency. MD simulations reveal marked differences in the intermolecular interactions in SST and ncSST providing plausible explanation for differential aggregation and fibril reversibility observed experimentally in these structural variants. Our findings thus emphasize that subtle changes in native structure of peptide hormone(s) could alter its conformational dynamics and amyloid formation, which might have significant implications on their reversible storage and secretion.

Protein/peptide hormones secreted through the regulated secretory pathway are known to be stored in a highly concentrated form within specialized membrane enclosed structures, known as ‘secretory granules’ (1-3). Owing to the intricate regulation of storage and secretion, the stored form of these hormones must possess properties of stability as well as reversibility. The exact structural form of these aggregates within the granule is thus of great interest. Recently, it was suggested that peptide/protein hormones under the regulated secretory pathway are stored in the form of amyloid-like structures within the secretory granules (4).

Amyloids are highly ordered protein/peptide aggregates that have conventionally been associated with diseases like Alzheimer’s, Parkinson’s and type 2 diabetes mellitus (5). However, increasing examples of amyloid-like structures in the non-disease context suggests that amyloid formation might be a generic property of proteins/peptides (6). Studies focusing on ‘functional amyloids’, i.e. amyloids involved in native biological functions have thus grown in recent times. Examples include the curli protein fibrils (7) of E. Coli that aid in surface attachment and colonization of the bacteria and the amyloid-like form of mammalian protein pmel17 that is used as a template for synthesis of pigment melanin (8). Augmenting our understanding of the potential functional role of amyloids in mammalian systems was the finding that various peptide/protein hormones showed the ability to form amyloid under granule relevant conditions in vitro (4). Further, the reversible nature of these hormone aggregates has also been reported (4,9). However, the ‘factors’ modulating hormone aggregation and reversibility of these aggregates must be understood to elucidate the mechanism(s) by which amyloids are utilized as a storage depot for the hormones. Additionally, the correct post-translation modification of proteins within the Golgi apparatus is important in maintaining their native structure. This could be crucial for the regulated formation of amyloid and subsequent release of the hormones. One of the essential factors governing protein/peptide structure and stability is the disulfide bond (10-12). The role of disulfide bond on structure, oligomerization and amyloidogenicity of several proteins relevant to diseases/biological functions has previously been postulated by various research groups (10,13-15).

In the current study, we chose a representative peptide hormone somatostatin-14 (Fig. 1), which is targeted to regulated secretory pathway, as a model system to understand the role of disulfide bond (Cys\(^3\)-Cys\(^{14}\)) in controlling its conformational flexibility, self-association and fibril disassembly (monomer release) profiles. Somatostatin-14 (SST-14) is a small cyclic peptide hormone secreted by hypothalamus, one of the well-known functions of which is to inhibit the release of growth hormone (16). Other important functions of somatostatin-14 include inhibition of gastrin and gastric-acid secretion, inhibition of insulin and glucagon secretion in pancreas, and
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regulation of amyloid-β peptide, Aβ42 in brain (17-19)

Somatostatin-14 has previously been reported to form amyloid fibrils in vitro (4,20). In this study, we first show that somatostatin-14 is stored as amyloid-like aggregates in rat hypothalamus tissues (Fig. 2). Next, to understand the role played by disulfide bond in modulating the structure of somatostatin, we performed structural analysis of two forms of the peptide; the disulfide bonded form (SST-14) and the disulfide reduced ‘non-cyclic’ form (ncSST-14). Although ncSST has previously been shown to exist endogenously within cells (21), its biological implication is not yet demonstrated. Our NMR and MD simulation results reveal significant difference in conformational flexibilities of the native (cyclic) and non-cyclic form of the peptide. Interestingly, our in vitro aggregation studies display accelerated aggregation kinetics of ncSST-14 compared to SST-14 in presence of a secretory granule relevant glycosaminoglycan, heparin (22-24). Further, our in silico aggregation studies reveal marked variation in interpeptide hydrogen bonding network upon cleavage of the disulfide bridge. The simulations also reveal the involvement of almost the entire peptide in self-association, which is further supported by our H/D exchange and proteinase-K digestion results. Finally, we observed considerable differences in fibril reversibility and resistance to thermal or chemical denaturation of the SST and ncSST fibrils, with the latter showing slower monomer releasing potency and increased resistance to denaturing conditions.

The current study not only underlines the pivotal role of disulfide bridge(s) in regulation of protein/peptide amyloid formation and the reversibility of the aggregates, but also provides the detailed mechanism of somatostatin amyloid formation, which has substantial value for understanding its storage within secretory granules.

EXPERIMENTAL PROCEDURES

Peptides and reagents- Somatostatin-14 used for the aggregation studies was purchased from BACHEM (Bubendorf, Switzerland). Other chemicals and reagents used were purchased from Sigma and Calbiochem. Water was double-distilled and deionized using a TKA Lab Tower AFT (Niederelbert, Germany).

Amyloid formation-One milligram of somatostatin-14 was dissolved in 500 µl of 5% D-mannitol, 0.01% sodium azide, and divided equally into two microfuge tubes such that the final peptide concentration was ~1200 µM. To one of these fractions, the glycosaminoglycan heparin (from a 10 mM stock in 5% D-mannitol, 0.01% sodium azide) was added such that the resulting heparin concentration was 400 µM. Thus, the peptide:heparin ratio in solution was finally 3:1. For the preparation of non-cyclic somatostatin, dithiothreitol (DTT) was first added to the peptide with a final concentration of 5 mM, and incubated overnight. Heparin was further added to the non-cyclic peptide in ratio as mentioned above. The solutions were incubated at 37°C and at the regular intervals, Thioflavin T (ThT) fluorescence and circular dichroism was performed to monitor the amyloid fibril formation and EM was done to confirm the morphology of fibrils. All the biophysical characterization experiments including Circular dichroism, Fourier Transform Infrared spectroscopy, ThT fluorescence and Nuclear Magnetic Resonance were performed at 25°C.

Thioflavin T fluorescence-The amyloid formation was monitored by binding and fluorescence intensity changes of the β-sheet sensitive dye, ThT. 2 µl of ThT solution (stock 1 mM) was added in 200 µl of diluted sample (final peptide concentration 15 µM). The ThT fluorescence was measured immediately in 10 mm path length quartz cuvette cell (Hellma, Forest Hills, NY) on a spectrofluorimeter (Shimadzu-RF-530) with an excitation wavelength 450 nm and emission spectra were recorded between 460 nm to 500 nm. The excitation and emission slit width was 5 nm for all the studies. ThT control in the experiment contained 2 µl ThT dye solution (1 mM) in 200 µl of D-mannitol. ThT fluorescence at 482 nm was plotted against incubation time.
Circular dichroism (CD)-Circular dichroism was performed to monitor the secondary structural transition during the course of SST aggregation. For CD, 5 µl of incubated peptide sample was taken and diluted up to 200 µl with 5% D-mannitol (final peptide concentration 30 µM). Sample was transferred into 0.1 cm path-length quartz cell (Hellma, Forest Hills, NY) and spectra were obtained using JASCO spectropolarimeter (model J-180). All measurements were done at 25ºC. Spectra were recorded over the wavelength range of 198-260nm. Three independent readings were taken with each sample. Raw data was processed by spectra smoothing and subtraction of D-mannitol spectra. Only heparin (10 µM) did not show any significant CD signal compared to peptide during the entire incubation period (data not shown). CD results were represented in molar ellipticity (kdeg dmol⁻¹ cm²).

Fourier transform infrared spectroscopy (FTIR)-FTIR is used for the determination of protein/peptide secondary structure. For FTIR analysis, thin translucent pellet of KBr was made by compressing the ground potassium bromide (KBr) powder at the pressure of 7 ton by using hydraulic pressure pump. The pellet was then kept under IR lamp and 5 µl of the SST-14 solution (1200 µM) was spotted on it and dried immediately. For background spectrum, 5 µl of the 5% D-mannitol was spotted on another KBr pellet and dried. The pellet was then kept in a transmission holder and the IR spectra in the range of 1800-1500 cm⁻¹ were acquired by using BrukerVertex-80 instrument equipped with DTGS detector. For each spectrum, 32 scans at the resolution of 4 cm⁻¹ were recorded. Raw data corresponding to amide-I region (1700-1600 cm⁻¹) were deconvoluted by Fourier Self Deconvolution (FSD) method. The deconvoluted spectra were then subjected to Lorentzian curve fitting procedure by using OPUS-65 software.

Tryptophan fluorescence-Tryptophan (Trp) fluorescence is used to probe changes in the microenvironment of the Trp residue in the proteins/peptides (25), and thus utilized for monitoring the protein folding and aggregation (26,27), (28). For tryptophan fluorescence, 1200 µM stock of peptide was diluted with 5% D-mannitol such that final protein concentration 15 µM. Sample was placed in 10 mm path length quartz cuvette cell (Hellma, Forest Hills, NY) and spectra were acquired using spectrofluorimeter (Shimadzu-RF-530). The spectra were recorded with an excitation wavelength 280 nm and emission wavelength range 290 nm–500 nm. The excitation and emission slit width was 5 nm for all the studies.

The time-resolved Trp fluorescence intensity decay kinetics experiments were carried out using a rhodamine 6G dye laser (Spectra Physics, Mountain View, CA), pumped by an Nd:YAG laser (Millenia X, Spectra Physics) and a time-correlated single-photon counting (TCSPC) set up coupled to micro-channel plate photomultiplier (model R2809u; Hamamatsu Corp). Pulses (1ps duration) of 885 nm radiation from the laser were frequency tripled to 295 nm by using a frequency tripler (GWU, Spectra physics). Each sample (100 µM) was excited at 295 nm at a pulse repetition rate of 4 MHz. For each sample, the emission was measured at their emission maxima (λ_max) as determined from Trp fluorescence spectra. The instrument response function (IRF) was obtained at the excitation wavelength using a dilute colloidal suspension of dried non-dairy coffee whitener. The width (full width at half maximum) of the IRF was ~40 ps. Peak counts of 10,000 were collected with the emission polarizer oriented at the magic angle (54.7°) with respect to excitation polarizer. A three-exponential function was used to fit the time-resolved fluorescence intensity decays. The mean fluorescence lifetime (τ_m) was calculated using τ_m = ∑ α_i τ_i, where α_i is the amplitude associated with each fluorescence lifetime τ_i and ∑ α_i = 1.

Acrylamide Quenching- Acrylamide quenching was carried out to determine relative solvent exposure of tryptophan in somatostatin before and after amyloid formation. Somatostatin monomer/fibril (100 µM) was prepared in 5% D-mannitol containing 0.01% (w/v) sodium azide. Peptide samples (150 µl) were mixed with increasing concentrations of acrylamide (0-0.25
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M) from a 5 M stock solution of acrylamide. For each quencher concentration, fluorescence lifetime measurements were performed as described in the previous section. The ratio \( \frac{\tau_0}{\tau} \) was calculated and plotted against concentrations of acrylamide \([Q]\). \( \tau_0 \) and \( \tau \) are the mean fluorescence lifetime in absence of acrylamide and that in presence of different concentrations of acrylamide, respectively. Stern-Volmer equation (Equation 1) was utilized to calculate the \( k_q \), bimolecular rate constant of dynamic quenching \((M^{-1}s^{-1})\).

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\frac{\tau_0}{\tau} = 1 + k_q \tau_0 [Q] \tag{1}
\]

N-acetyl tryptophan amide (NATA) was used as standard reference control for the fluorescence quenching studies. In case of ncSST samples, as DTT was used to reduce SST to ncSST, DTT was added in the standard reference sample (NATA+DTT).

Transmission Electron Microscopy (TEM)-To characterize the morphology of the somatostatin aggregates, transmission electron microscopy was performed at various time points during aggregation till amyloid formation was completed. A 2.5 μl aliquot of protein of 1200 μM sample was diluted to 75 μl with 5% D-mannitol, so that the final protein concentration was ~40 μM, spotted on a glow-discharged, carbon-coated Formvar grid (Electron Microscopy Sciences, Fort Washington, PA), incubated for 5 min, washed with distilled water, and then stained with 1% (w/v) aqueous uranyl formate solution. Uranyl formate solution was freshly prepared and filtered through 0.2 micron sterile syringe filter (Millex™, MILLIPORE, Bedford, USA) before use. The imaging of samples was performed using a transmission electron microscope (TECNAI12 D312 FEI, Netherlands) at 120 kV with nominal magnifications between 26,000 X and 43,000 X. Images were recorded digitally using the Megaview imaging system.

Congo red birefringence-Somatostatin solutions (1200 μM) were incubated at 37°C with slight agitation until fibrils were formed. Somatostatin fibrils (SST and ncSST) were isolated by ultracentrifugation (90,000 rpm for 45 mins) and stained with alkaline Congo Red (CR) dye (100 μM CR in PBS containing 10 % ethanol) for 15 minutes and spread evenly on glass slide. After drying under vacuum, the slides were analyzed using a light microscope (Olympus SZ61 stereo zoom microscope, Japan) equipped with two polarizers. The images were first viewed under bright field and followed by observation under cross-polarized light. Those regions that display CR binding (red) were analyzed under cross-polarizer and images captured using attached CCD camera.

Congo red absorbance-The peptide fibril/monomer (5 μl) was diluted with 80 μl of PBS buffer (containing 10% ethanol). Then, 15 μl of a 0.1 mM Congo red solution prepared in PBS containing 10% ethanol was added. The solution was used for UV absorbance measurements in the range 400-600 nm. The JASCO V-650 spectrophotometer was used for this study. 15 μl of CR solution added to 85 μl of PBS containing 10% ethanol was used as a control.

Monomer release assay-Amyloid fibrils of the SST and ncSST were tested for their ability to release their monomer counterparts. For this, 100 μl of 400 μM of the fibrils in 5% D-mannitol, 0.01% sodium azide were subjected to dialysis through a 3.5 kDa cut-off membrane against 500 μl of Tris HCl buffer (pH 7.4), 0.01% sodium azide. The fibril samples were placed into a Slide-A-Lyzer mini dialysis unit system (PIERCE, Rockford, IL, USA), capped and positioned into a 1 ml cryo tube (Nunc, Denmark) containing 500 μl of Tris HCl buffer (pH 7.4), 0.01% sodium azide. The Slide-A-Lyzer dialysis unit was placed such that its membrane was in contact with the Tris buffer solution. Magnetic bars were kept into individual tubes and the assembled units were placed on a magnetic stirrer (Spinot, Jaibro Scientific Works, New Delhi, India). The rotation of magnetic bars provided the constant movement in the outer solution further allowing monomer release. After suitable time intervals (0, 3, 6, 12, 24, 36 and 48 hrs), the solution from outside the dialysis membrane was taken for analysis. To monitor the monomer release, an aliquot of 150 μl solutions from the cryo-tube (outside the
membrane) was taken and tryptophan fluorescence was measured. The outside solution was returned to the cryo-tube and the dialysis system was reassembled after each reading.

Denaturation study of fibrils- The fibrils of cyclic or non-cyclic somatostatin-14 were subjected to chemical and thermal denaturation to compare their relative tolerance to denaturing conditions. Chemical denaturation was carried out by incubating the 30 μM fibrils from each sample in presence of increasing concentrations of GdnHCl (0M, 0.5M, 1M, 1.25M, 1.5M, 1.75M, 2M, 3M, 4M and 6M) prepared in 5% D-mannitol containing 0.01% sodium azide. The solutions were incubated overnight for equilibrium unfolding at room temperature. Trp fluorescence of these samples was recorded for each concentration of GdnHCl. The shift in wavelength corresponding to maximum fluorescence intensity (λ\(_{\text{max}}\)) was plotted against each GdnHCl concentration. For thermal denaturation study, the 30 μM SST or ncSST fibrils prepared in 5% D-mannitol containing 0.01% sodium azide were placed in spectrophuorimeter attached with temperature control system. Samples were then subjected to an increasing temperature gradient (25°C-95°C) with 10°C increment and Trp fluorescence of these samples was recorded at each temperature. The shift in Trp λ\(_{\text{max}}\) was plotted against each temperature. All spectra were acquired using a spectrofluorimeter (Fluoromax-4, Horiba Scientific).

Proteinase K digestion- To determine the ‘core’ of the somatostatin amyloid, the preformed fibrils were subjected to proteinase K digestion, owing to the fact that the ‘amyloid-core’ is resistant to enzyme digestion. Both fibrils and monomers (100 μM) were separately treated with 20 μg/ml proteinase K and incubated at 37 °C. At different time points (10 mins, 20 mins, 30 mins, 45 mins, 60 mins, 80 mins and 480 mins), the digestion profiles of the treated fibril samples relative to monomers were analyzed by mass spectrometry (Autoflex Speed, Bruker MALDI-TOF-TOF mass spectrometer; mass range 1100-1800; mass tolerance 3 Da). The spectra obtained at different time-points were plotted after normalizing peak intensities with respect to highest intensity peak (base peak) in each spectrum.

Hydrogen/Deuterium exchange of somatostatin fibrils- To determine the involvement of amide hydrogens in both forms of SST-14 fibrils, H/D exchange experiment was performed. For this experiment, SST was dissolved in 5% D-mannitol (containing 0.01% NaN\(_3\)), such that the final concentration of the peptide was 1200 μM. Heparin was added to the peptide solution to allow fibril formation where peptide:heparin molar ratio was 3:1. Solutions were incubated at 37°C with slight agitation until fibrils formation. Amyloid fibrils were confirmed by high ThT binding and appearance of fibrillar morphology using TEM. Similarly, the ncSST fibrils were prepared in presence of heparin. The SST or ncSST fibrils were then pelleted down by ultracentrifugation at 90,000 rpm for 45 mins at room temperature. The fibril pellet was resuspended in 5% D-mannitol, 0.01% sodium azide and again pelleted down by ultracentrifugation (wash). The washing step was repeated twice and the fibril pellet was retrieved. This pellet was then thoroughly resuspended in 5% D-mannitol and equal volumes of the resuspension were aliquoted into two microfuge tubes. The tubes were quickly frozen in liquid nitrogen, and were subjected to lyophilization. The lyophilized fibrils were used for the H/D exchange. One of the samples was added with autoclaved distilled water, and the other sample was treated with D\(_2\)O. The D\(_2\)O treated sample was kept under slight rotation (50 rpm) at room temperature, allowing H/D exchange, and was quick frozen in liquid nitrogen to stop the H/D reaction after 8 days (HD8). The water treated sample was quick frozen on day-0 itself (HD0). Both the frozen samples were then lyophilized. To determine the H/D exchange, the lyophilized samples (fibrils) were dissolved in DMSO-d6, 2.5% TFA and NMR experiments were performed.

NMR Spectroscopy- All ^1H NMR experiments were performed on a Bruker Avance 500 MHz NMR spectrometer equipped with triple resonance gradient probe at 298K. Data were processed using
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Topspin 2.1 version and analyzed with Sparky 3.114. DSS (4,4-dimethyl-4-silapentane-1-sulfonic acid) was used as an internal reference for calibration of proton chemical shifts. Spectra of peptides (SST and ncSST) were collected at a concentration of ~600 μM in a H₂O/D₂O (90:10) containing 5% D-mannitol. Series of one-dimensional spectra of peptides were recorded with 1024 scans and 16K data points in presence and absence of heparin. For sequence-specific resonance assignments, two-dimensional (2D) TOCSY with MLEV17 sequence with a mixing time of 60 ms - 80 ms and NOESY spectra with mixing times of 200 ms were recorded. For H/D exchange experiments, the solvent used was DMSO-d6 and the mixing time was 80 ms and 200 ms for TOCSY and NOESY, respectively. All 2D experiments were acquired with 60 scans having (2048x320) data matrix with an acquisition time of 17.04 ms and 2.66 ms during direct and indirect dimension. The data were zero-filled to give a (4096x1024) matrix and processed prior to Fourier transformation with sine square bell. In all experiments, suppression of the water signal was achieved with the Excitation Sculpting scheme prior to acquisition.

Structure calculation-Structure of SST-14 in presence of heparin in 5% D-Mannitol was calculated, whereas in absence of heparin, NOESY signals could not be obtained, which disallowed calculation of its structure. The structural restraints for the somatostatin-14 peptide were obtained based upon NMR derived NOEs while the dihedral constraints were obtained using the GRIDSEARCH and FOUND (HABAS) macro of CYANA 2.1(29), which converts the upper distance limits into torsion angle restraints. We utilized 79 NOE constraints and 47 angle constraints in order to calculate the conformers (Table S1). Initially, 100 conformers were generated using simulated annealing protocol in the program CYANA and 10 of those conformers with the lowest target function values were utilized for energy minimization using molecular dynamics software package NAMD (30) and the CHARMM 2.7 forcefield (31,32). Energy minimization was performed using a stepwise steepest descent algorithm followed by the conjugate gradient algorithm for 15,000 steps. The energy minimized structures were then validated using Ramachandran Plot assessment program RAMPAGE (33) (Table S1). The resulting conformers were then equilibrated in a TIP3P explicit water box for 200 ns and were finally used as a basis of the solution structure of somatostatin-14. The starting structure for studying conformational dynamics of ncSST in silico was modeled using the NMR structure of SST-14 with cleaved disulfide bond (Cys³-Cys¹₄). The conformational dynamics of the two forms of the peptide was then assessed from the 200 ns trajectories. The structures and the molecular dynamics trajectories were analyzed using PYMOL (34) and VMD (35). The coordinates and NMR restraints of the resolved structure have been deposited in Protein Data Bank (PDB ID: 2MI1) and Biological Magnetic Resonance Bank (BMRB accession number: 19663).

MD simulations-All atom molecular dynamics (MD) simulations were performed to characterize the conformational dynamics of the SST and ncSST, using structures resolved by NMR spectroscopy. The simulations were performed using molecular dynamics software package NAMD 2.9 (30) and the CHARMM 2.7 (31) protein force field. The trajectories were analyzed using CARMA (36) trajectory analysis package. The various conformations accessed by the peptide were assessed by clustering them into conformations with RMSD within a tolerance range in software package CHIMERA (37). The most frequently accessed states and the frequency with which they are accessed were reported.

To study the aggregation propensity of somatostatin and the role of heparin in promoting their aggregation, 24 molecules of the peptide were simulated in the presence and absence of heparin for 100 ns each. The initial conformations of the 24 peptides in the simulation box were chosen according to the frequencies with which the monomeric peptide accessed various states. These trajectories were analyzed for their interpeptide hydrogen bonding, peptide-heparin hydrogen bonds using VMD (35). The snapshots
were rendered using PyMol (34). The simulations were performed with an electrostatic cut-off of 12 Å units, a van der Waals cut-off of 10 Å units along with periodic boundary conditions. The systems were first energy minimized and then heated to 310 K with gradual scaled increase in temperature. This was followed by a constant pressure equilibration for 3 ns and then a 100 ns production run in NPT. All simulations were performed till the simulations reached convergence.

Immunofluorescence-In order to characterize the nature of somatostatin aggregates within secretory granules, rat hypothalamus tissues were immunohistochemically analyzed. Adult, male, Sprague-Dawley rats (200-250 g) were used. Animals were maintained under 12 h light:12 darkness cycle maintaining the standard temperature and humidity of the animal facility. The food and water was provided ad libitum. All experimental protocols were reviewed and approved by the Institutional Animal Ethical Committee (IAEC) at NISER, Bhubaneswar, India. Rats were deeply anaesthetized with intraperitoneal administration of mixture of ketamine (100 mg/kg) and xylazine (10 mg/Kg), and perfused transcardially with ice-cold phosphate buffered saline (PBS, pH 7.4) followed by 50 ml 4% paraformaldehyde in phosphate buffer (PB, pH 7.4). The brains were removed, post-fixed in 4% paraformaldehyde followed by cryoprotection in 25% sucrose in PBS overnight, rapidly frozen on dry ice, mounted using Tissue-Tek in cryostat, and a block containing the hypothalamus was isolated. Serial 20 µm thick coronal sections through the rostro-caudal extent of the hypothalamus were cut on a cryostat (Leica CM3050S, Leica Microsystems, Nussloch GmbH, Germany), collected on poly-l-lysine (Sigma) coated glass slides, and stored at -20°C until processed further. Before proceeding for immunostaining, slides were kept at room temperature for 15 mins. The sections were first washed with phosphate-buffered saline (PBS), pH 7.4 twice. Antigen retrieval was performed on the sections by treating them with 0.5% Triton X-100 (in PBS) for 20 mins at room temperature. The sections were next washed with PBST (PBS with tween-20, pH 7.4). Non-specific antigenic sites were blocked using PBST containing 2% bovine serum albumin (BSA). The sections were sequentially immuno-stained, first with rabbit polyclonal somatostatin antiserum (Chemicon, USA) at 1:1000 dilution and then with anti-amyloid OC antibody (rabbit polyclonal, Millipore, 1:1000), with respective secondary antibody staining after each primary antibody. While the sections were incubated in primary antibodies overnight at 4°C, secondary antibody incubation was carried out at room temperature for 1h. For SST and amyloid staining (using OC antibody), AlexaFluor647-conjugated (red) and AlexaFluor488-conjugated (green) secondary antibodies (Invitrogen) were used, respectively. The sections were rinsed with PBST, mounted with mounting media containing 1% DABCO (Sigma) in 90% glycerol and 10% PBS and visualized under Olympus IX 81 confocal microscope. For Thio S staining, anti-SST immunostained sections were first incubated with AlexaFluor647-conjugated secondary antibody. The sections were rinsed with PBST and treated with 1% Thioflavin S (Thio S) (Sigma Aldrich) for 10 mins in dark. The sections were then washed twice with 50% ethanol-PBST followed by PBST. Sections were mounted and observed under a confocal microscope.

RESULTS

Somatostatin-14 is a fourteen residue peptide hormone, where Cys³ is disulfide bonded with Cys¹⁴ to form a cyclic structure. Somatostatin has been shown to form amyloid fibrils in vitro, suggesting that amyloid could be the storage state of SST in secretory granules (4,38). To substantiate this finding within mammalian tissue, we performed immunohistochemical (IHC) studies using rat hypothalamus tissue sections. We utilized the rabbit polyclonal somatostatin antiserum and OC antibody (or Thio S dye) for somatostatin and amyloid detection within tissues, respectively. Application of somatostatin antisera (raised against somatostatin-14 peptide as well as
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somatostatin) from two different sources (Chemicon and Abcam, USA) resulted in similar immunostaining pattern in the hypothalamic periventricular neurons. Furthermore, the somatostatin antibody used in the present study has recently been used for the localization of somatostatin neurons in the periventricular nucleus in the hypothalamus (39). Results of the current study showing co-localization of SST and Thioflavin S (or OC antibody) staining in hypothalamic tissues suggest that SST is stored within the secretory granules as amyloids (Fig. 2).

To investigate the role of native structure in the regulation of amyloid formation and its subsequent monomer release (from fibrils), we studied both native cyclic somatostatin (SST) and disulfide-reduced non-cyclic somatostatin (ncSST) using various biophysical and computational approaches. The glycosaminoglycan heparin was used as an ‘aggregation inducer’ in our studies, as it has been suggested to accelerate protein aggregation and regulate protein/peptide hormone aggregation associated with their secretory granule biogenesis (9,22,23,40,41).

In vitro aggregation of somatostatin-14-Far UV-CD spectroscopy (198 nm to 260 nm) was used to monitor the possible conformational changes in SST during aggregation. As evident from the CD spectra, SST initially displayed unstructured conformation (random coil) since it showed negative minima at ~198 nm (data not shown). However, significant secondary structural change was observed during the course of aggregation (Fig. 3A) in presence of heparin. After 6.5 hrs of incubation, a conformational change was observed from random coil to a ‘helix-rich’ structure displaying CD spectra with two distinct minima one at ~208 nm and another at ~222 nm (Fig. 3A). The helix-rich state however did not further convert to classical β-sheet structure (single minima at ~218 nm) after 15 days incubation. Instead it transitioned to a secondary structure, where CD spectra showed two minima one at ~215 nm and another at ~240 nm after fibril formation. The observation of two negative minima in SST amyloids has been reported earlier (4). Moreover, it was previously shown that the disulfide bond chromophore can give rise to a negative minima at 240 nm in L-Cystine crystals (42). Therefore, the peak at ~240 nm for SST fibrils could be due to the alignment/ordering of disulfide bond after fibrillation. In the absence of heparin, SST mostly remained in unstructured conformation (random coil) throughout the period of incubation (data not shown).

The amyloid-specific dye Thioflavin T (ThT) was utilized to monitor the kinetics of SST amyloid formation. ThT has been shown to bind specifically to cross-β sheet structures of amyloids (43,44), thus routinely used to monitor amyloid formation kinetics (45-48). At the beginning of the experiment (0 hrs), low ThT binding was observed in both samples (SST or SST-Hep) (Fig. 3B). However, SST in presence of heparin showed increase in ThT binding after 3 hrs of incubation, which reached the maximum at ~6.5 hrs (Fig. 3B). After 13 hrs of incubation and thereafter, ThT fluorescence intensity steadily decreased and then remained constant after 72 hrs of incubation. The enhanced ThT fluorescence during the course of incubation indicates the formation of amyloid-like structures by somatostatin. The decrease in ThT fluorescence at later time points perhaps was due to inaccessibility of the ThT binding sites in the higher ordered ‘bundled-packs’ of SST fibrils (Fig. 3F, 360 h). There was no appreciable change in ThT fluorescence intensity observed in somatostatin control (without heparin) with prolonged incubation time (Fig. 3B).

We also utilized tryptophan (Trp) fluorescence to evaluate structure-specific changes during SST fibrillation. A time-dependent blue shift in wavelength corresponding to Trp fluorescence maxima (λmax) was observed during aggregation (~348 nm to ~337 nm) when SST was incubated in presence of heparin (Fig. 3C). Additionally, a gradual decrease in Trp fluorescence intensity was observed during SST fibrillation (Fig. 3C). The low quantum yield of Trp in the fibril state might be due to the effect of potential quencher residues in the vicinity of Trp at this state and/or precipitation of SST fibrils. In the absence of
heparin, the Trp fluorescence of SST remained unaltered even after 360 hrs of incubation (Fig. 3C).

We further studied the microenvironment of Trp in monomer and fibrils using time-resolved fluorescence intensity decay kinetics (Fig. 3D). All the decay kinetics were fitted to a three exponential function and the mean lifetime ($\tau_m$) values derived showed lesser fluorescence lifetime for SST fibrils ($\tau_m=0.64$ ns) compared to SST monomers ($\tau_m=1.71$ns). The data indicate an alteration of Trp microenvironment upon amyloid formation in SST. In addition, we performed dynamic quenching of Trp fluorescence using lifetime measurements to delineate solvent exposure of Trp before and after amyloid formation (Fig. 3E). It was interesting to observe that the $k_q$ values of SST ($k_q=6.27 \times 10^9$ M$^{-1}$s$^{-1}$) and SST fibrils ($k_q= 9.07 \times 10^9$ M$^{-1}$s$^{-1}$) were of the same order of magnitude as that of NATA ($k_q= 9.49 \times 10^9$ M$^{-1}$s$^{-1}$). The standard deviation in the values of fluorescence lifetimes and $k_q$ were about 5-10 % of the values.

Overall, these results suggest that irrespective of a different microenvironment experienced by Trp in the amyloid state compared to monomer, the solvent exposure of Trp in monomeric Vs fibrillar state is not significantly altered. Although there is a blue-shift of $\lambda_{max}$ observed during aggregation, a similar extent of solvent exposure in monomeric and fibrillar state indicates that the Trp gets only partially buried during amyloid formation.

To examine the morphological development of SST during aggregation, peptide solution was analyzed during the incubation using TEM. Immediately after dissolution and addition of heparin, SST showed mostly amorphous oligomers with sparsely populated thin filaments. However, major population of filamentous fibrils (fibril width ~10 nm) was observed in SST incubated in presence of heparin after ~6.5 hrs. These structures transformed into slightly thicker and straight fibrils (Fig. 3F, 13-120 h), which eventually formed thick fibrillar bundles at the end (Fig. 3F, 360 h) of the study. These mature fibrils were composed of several laterally associated filaments, which range between 10 and 12 in numbers. We observed no fibrillar structures in SST in absence of heparin after incubation up to 360 hrs (data not shown). Although SST formed amyloid fibrils, displaying increased ThT binding, these did not show a traditional $\beta$-sheet conformation in CD (Fig. 3A). To confirm the $\beta$-sheet conformation, FTIR study was performed. The FTIR data with fibrils showed a peak ~1638 cm$^{-1}$ suggesting presence of $\beta$-sheet content in the fibrils (Fig. 3G). Further, the SST fibrils exhibited apple-green birefringence upon Congo red staining under cross-polarizer (Fig.3H) and increased CR absorbance (Fig.3I) indicating their amyloidogenic nature.

Non-cyclic somatostatin-14 forms amyloid fibrils instantaneously-To probe the role of disulfide bond of somatostatin-14 in its aggregation, ncSST was incubated in presence and absence of heparin. The ncSST was random coil initially as shown by single minima at ~198 nm in CD (data not shown), however after 12 mins of incubation in presence of heparin, the peptide showed transition to helix-rich state (two minima in CD spectra at ~208 and ~222 nm). After 20 mins of incubation, it converted to $\beta$-sheet rich structure (negative minima at ~216) (Fig. 4A), which remains mostly unaltered up to 3 days of measurement. The data indicates the rapid amyloid formation by ncSST. Consistent with CD data, ThT binding study also showed significant increase in fluorescence from the beginning of the aggregation reaction itself in presence of heparin, which did not change during first 20 mins of incubation and fibril formation. However, after 20 mins, ThT fluorescence gradually increased during fibrillation (Fig. 4B), which becomes stationary after 3 hrs of incubation. In absence of heparin however, ncSST neither showed any conformational-transition (data not shown) nor any substantial change in ThT binding during the entire incubation period (Fig. 4B). Additionally, Trp fluorescence was monitored during the course of aggregation and the data suggest that
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immediately after addition of heparin, a blue-shift (~352 nm to ~342 nm) in wavelength maxima (λ\text{max}) was observed (Fig. 4C), which remain unaltered during rest of the incubation period. We also observed increase in fluorescence intensity immediately after addition of heparin (at 0 min). However, during the course of aggregation, the Trp fluorescence intensity gradually decreased (Fig. 4C), which could be due to the partial precipitation and/or Trp fluorescence quenching by nearby residues.

The Trp microenvironment of ncSST in monomer and fibrils (72 hr old) were further probed by fluorescence intensity decay kinetics (Fig. 4D). The three exponential fit of the data and the derived mean fluorescence lifetime value suggest that Trp in fibrils possess lower life time (τ\text{m} = 0.79 ns), thus a different microenvironment compared to monomers (τ\text{m} = 1.89 ns). The results from dynamic fluorescence quenching using lifetime measurements (Fig. 4D) however reveal that the extent of solvent exposure of Trp in ncSST fibrils (kq = 7.72 x 10^9 M\textsuperscript{-1}s\textsuperscript{-1}) was of the same order of magnitude as that of ncSST monomer (kq = 5.83 x 10^9 M\textsuperscript{-1}s\textsuperscript{-1}) and NATA (in presence DTT) (kq = 9.81 x 10^9 M\textsuperscript{-1}s\textsuperscript{-1}). The standard deviation in τ\text{m} and kq were about 5-10 % of the values. Collectively, the fluorescence data suggest that only a partial burial of tryptophan occur during fibrillation of ncSST similar to SST fibrillation.

Electron microscopic studies for the morphological characterization of ncSST fibrillation displayed thin fibrillar structures in samples at early time-points (Fig. 4F, 6 mins). Electron micrographs at different time-points during aggregation indicated the presence of long, flexible filamentous fibrils (~6-12 nm width) of ncSST (with hep) (Fig. 4F), whereas only small oligomeric forms with no defined morphology were seen in absence of heparin (data not shown). Consistent with CD data showing β-sheet secondary structure (negative minima at 216 nm), FTIR of the ncSST fibrils showed a major peak at ~1633 cm\textsuperscript{-1} (Fig. 4G), indicative of parallel β-sheet content in the amyloid. The amyloid nature of the ncSST fibrils was also confirmed by the display of apple-green birefringence under cross-polarizer upon Congo red staining (Fig. 4H) as well as increase in CR absorbance (Fig. 4I) in UV spectroscopy.

Overall, our \textit{in vitro} aggregation experiments suggest that the disulfide bond between Cys\textsuperscript{3} and Cys\textsuperscript{14} in SST-14 can significantly influence amyloid formation of the peptide.

**Initial structure of somatostatin leading to fibrillation**-Characterizing the underlying structural features of the cyclic and non-cyclic somatostatin contributing to their differential aggregation behavior is very crucial to understand ‘conformation-aggregation’ relationship. To structurally characterize the monomeric somatostatin peptide (cyclic and non-cyclic), we used NMR spectroscopy both in presence and absence of heparin. We performed \textsuperscript{1}H NMR experiments of SST to investigate its monomeric structure in water (containing 5% D-mannitol). The 1D proton spectrum of SST monomer showed distinct and sharp peaks, which shifted to lower ppm values upon addition of heparin (Fig. 5A), indicating subtle structural changes in the peptide. The peaks in the latter case also appeared relatively broader. Our TOCSY (2D) spectrum also showed the same result, wherein the peak shifts of individual amino acids upon heparin addition were clearly observed (Fig. 5B). Due to absence of long-range NOESY cross-peaks, the structure of SST monomer could not be calculated. However, heparin addition allowed the peptide to assume a definite structure, the TOCSY and NOESY peaks of which were used to determine its structure. The NOESY spectrum in Fig. 5C shows the long range and medium range NOEs used for structure calculation. The structure calculation was performed, as discussed in Experimental Procedures section. An ensemble of 10 structures of SST (Fig. 5D) was obtained using CYANA program, and the average structure from the ensemble is represented in Fig. 5E. We find the structure of somatostatin-14 determined in this work to be comparable to already reported structures of SST analogs (49).

We also performed \textsuperscript{1}H NMR experiments with ncSST (in 5% D-mannitol) to resolve its structural
characteristics. In the absence of heparin, ncSST showed distinct peaks in the 1D proton spectra that disappeared instantaneously upon addition of heparin. Furthermore, peak broadening was seen in this case suggesting the formation of higher order structures immediately upon heparin addition (Fig. 5F). Only few peaks indicative of side chains from aromatic amino acids were visible in ‘ncSST+Hep’ sample (Fig. 5F, bottom). The chemical shift assignments of proton resonances in ncSST were carried out using the two-dimensional NMR experiments (Fig. 5G). We could not however assign the resonances corresponding to cysteine residues (C3 and C14) in the peptide. TOCSY data although aided assignment of most of the amino acids in ncSST, the experimental NOEs were obtained only for sequential amino acids (Fig. 5H) and therefore did not yield meaningful distant restraints to determine the structure of ncSST in solution (water). This was further supported by the observation of near-zero secondary chemical shift indices displayed by most of the amino acid residues in ncSST (Fig. 5I).

Further, it was interesting to note that the TOCSY spectra of SST and ncSST showed significant differences (Fig. 5B and 5G). The reason for the observed differences could be attributed to a change in conformational ensemble properties of somatostatin resulting from an altered local environment of the amino acid residues in peptide upon release of the disulfide bridge.

Somatostatin-14 structure determined from this study was further analyzed using computational approaches. We utilized the NMR-derived structure for aggregation studies of somatostatin in silico.

**Structural dynamics of somatostatin-14 in silico**—To understand the time resolved properties of NMR-derived SST structure, classical MD simulations were performed in explicit solvent. All-atom MD simulations are powerful tools to study protein structural dynamics (50,51) and aggregation (52-54). Our MD simulation results reveal the tendency of SST to assume predominantly two structures with varying frequencies as evident from the distribution of the radius of gyration of the peptide main chain during the simulation (Fig. 6A, SST). The peptide also accesses other states however with relatively low frequencies (Fig. 6B, SST, 3, 4 and 5). The non-cyclic form of somatostatin (ncSST) however displayed a largely multi-state dynamics (Fig. 6A, ncSST) suggesting that the cleavage of the disulfide bond had a major effect on the conformational flexibility of the peptide. It was interesting to note that ncSST assumes various states in solution, where the most frequently accessed state showed the higher helicity. However, the peptide accesses this state (state 1 in Fig 6B, lower panel) with low probability (~0.08). Interestingly, the other states assumed by ncSST (i.e. states 2, 3, 4, 5 in Fig 6B, lower panel) also exhibit lower frequencies. The divergence observed in ncSST structures during the simulation was due to loss of native contacts with time. Additionally, both SST and ncSST tend to deviate away from the starting structure during the simulation reflecting on the loss of native contacts with time (Fig. S1A, top). Since we used NMR structure of SST determined in presence of heparin for the simulation of SST, the observed $R_g$ value changes (~7 Å to ~5.5 Å) during simulation could be due to the different conformation (more compact) the peptide assumes in the absence of heparin. The root mean square deviation (RMSD), radii of gyration, surface area and end-end distance profiles were smoother in case of SST while displaying a dynamic behavior in ncSST (Fig. S1A and S1B). Somatostatin thus demonstrates a significantly high conformational flexibility with vastly fluctuating profiles of reaction coordinates in the absence of the disulfide bond.

**Somatostatin aggregation in silico**—To delineate the similarities and differences in aggregation of SST and ncSST, 100 ns MD simulations of the peptides in explicit solvent were performed. The starting positions of the peptides in the four systems (SST, SST + heparin, ncSST and ncSST + heparin) were initially unbiased and randomly positioned to avoid any intermolecular contacts at the beginning of the simulation. The initial configurations of the systems can be visualized...
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from Fig. 7A (left) and Fig. 8A (left). It is interesting to note that both the cyclic and non-cyclic forms of the peptide showed very negligible self-association tendencies in the absence of the glycosaminoglycan heparin in the solvent box consistent with our in vitro data. However, addition of heparin was observed to significantly promote self-association of peptides in both SST and ncsST systems, as evident from the snapshot of the final state of the system in Fig. 7A (right) and 8A (right). A more detailed timeline of progression of the four systems and their higher self-association tendencies in presence of heparin is represented in Fig. S1C. Since the interpeptide contacts are one of the major driving forces for peptide/protein aggregation, we analyzed all the four simulation systems for interpeptide contacts during the simulation time-scale. Interestingly, both the cyclic and non-cyclic forms of somatostatin-14 in the absence of heparin showed negligible interpeptide hydrogen bond development during the course of the 100 ns simulation (Fig. 7B and 8B). However, the addition of heparin to the solvent box seemed to promote the self-association of both the peptides with an increase in interpeptide hydrogen bonding (Fig. 7B, 8B and S2A). Furthermore, while both SST and ncsST systems demonstrate increased heparin interactions during the simulation, the non-cyclic form of the peptide shows significantly higher interacting tendency with heparin (Fig. S2B). One of the measures of characterizing the self-association of the peptides is to monitor the cumulative surface area of the peptides over time. A gradual decline in the total surface area of SST and ncsST peptide systems over time in the presence of heparin indicates self-association of peptides during the simulation (Fig. 7D and 8D). Such a decline was however not observed in the absence of heparin in the solvent box, thereby enforcing the role of heparin in somatostatin aggregation. The self-assembling tendencies of the two peptides in the presence of heparin were further confirmed by a decrease in the Rg of the system during the simulation (Fig. 7E and 8E). We further analyzed the specific differences in the interpeptide hydrogen bonding network in the SST and ncsST systems to identify whether disulfide bond cleavage leads to differences in hydrogen bonding network in the aggregated state. A clear difference in hydrogen bonding patterns was observed in SST and ncsST. Whilst the most frequently observed hydrogen bonds in both the cases remained same (K4-C14 and K9-C14), there was a major change in the hydrogen bonding patterns of other residues involved in interpeptide contacts among the two somatostatin variants (Fig. 7C and 8C). The most prominent difference was the prevalence of hydrogen bonding between hydrophobic residues F6, F11 and W8 in ncsST (Fig. 8C). This was however not evident from the simulations of the SST system in presence of heparin (Fig. 7C). Also, while the residues F6-T12 are all involved as hydrogen bond donors in case of SST, the H-bonding pattern in case of ncsST shows the region K4-K9 acting as major H-bonding donors, while the C-terminus of the peptide (residues F11-C14) plays a key role of H-bonding acceptor (Fig. 8C).

Amyloid core of somatostatin fibrils-We performed H/D exchange (coupled with NMR) and proteinase K digestion (coupled with mass spectrometry) experiments with the fibrils to determine the involvement of peptide segment in SST and ncsST amyloid core formation. The fibrils were treated with proteinase K and the samples were analyzed by mass spectrometry at different time-points as mentioned previously (Experimental Procedures section). In parallel, only SST and ncsST monomer treated with the enzyme was used as controls. The SST fibrils upon proteinase K treatment did not show any digested product(s) up to 80 minutes. At this time point however, the SST monomer control showed two prominent fragments; peaks at 1277 Da (peptide fragment excluding K9-T10-F11) and 1404 Da (peptide fragment excluding F11-T12). We found that the peak corresponding to full length peptide 1655 Da (1637+1 H2O) totally diminished by this time (Fig. 9A, Monomer). The same peak however remained...
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unaltered in the SST fibrils, indicating that the entire stretch of the peptide is perhaps proteinase-resistant. After 480 minutes of enzyme reaction, the SST monomer displayed complete degradation (Fig. 9A, Monomer), while the fibrils showed only partial digestion at this time point (Fig. 9A, Fibril). Apart from the major peak 1637 Da corresponding to intact full-length SST peptide, the smaller peaks that were observed in fibril sample were 1277 Da and 1404 Da (Fig. 9A, Fibril, peaks a and b respectively). The digestion profiles of SST fibrils therefore suggest that the entire peptide in the fibrillar state is resistant to enzyme-mediated degradation, indicating that almost all amino acid residues in somatostatin are protected and form the ‘amyloid-core’. This is also consistent with the results of aggregation prediction algorithm Zyggregator (55) (Fig. 9B). To further delineate amyloid core at residue specific resolution, H/D exchange experiments coupled with NMR spectroscopy was carried out. The data showed that the intensity of SST amide protons of each amino acid was mostly unchanged even after subjecting the peptide fibrils to deuterium exchange for 8 days (Fig. 9C). The peak intensities of day-0 versus day-8 are shown in Fig. 9D, wherein we observe only a few peaks (G2, K9 and F11) showing slight reduction in intensities relative to other peaks after 8 days of H/D exchange.

Similarly, the proteinase K digestion of ncSST fibrils showed that ncSST in the fibrils did not get digested upon 480 mins of proteinase K treatment (Fig. 9E, left), as indicated by the intact 1638 Da peak corresponding to full-length ncSST in the fibrils throughout the reaction. In contrast, the ncSST monomer gets digested within 10 mins of the proteinase K reaction (Fig. 9E, right). The resultant peak (1350 Da) at 10 mins corresponds to ncSST fragment excluding the sequence T12-S13-C14. At later time points, the monomer gets completely digested. Our data therefore suggest that almost the entire peptide is involved in amyloid formation. The H/D exchange also show that most of the amide protons in the fibril state were protected from H/D exchange (Fig. 9F) except 3 peaks (G2, N5 and W8), which showed decrease in peak intensity (thus increased exchange) upon eight day incubation with deuterium in the solution (Fig. 9G). The partial H/D exchange of these residues after prolonged period might be due to the partial solvent exposure of these residues in the fibrillar state.

Monomer release and denaturation of SST versus ncSST fibrils-As both the cyclic and non-cyclic form of the peptide exhibited differences in conformation, aggregation and fibril characteristics; we were interested to study if these differences arising from structural change (S-S cleavage) in somatostatin can result in any variation in the ‘reversible’ property of the fibrils. Previous reports show that the hormone amyloids are reversible, thus can successfully release monomers under suitable experimental conditions (4,9). On this basis, we designed monomer release experiments by exposing the two different fibrils to physiological pH 7.4. SST or ncSST fibrils were allowed to release monomers by subjecting them to dialysis using a 3.5 kDa cut-off membrane and the peptide release was monitored by measuring Trp fluorescence of the solution outside the membrane at different time-points. We find that SST fibrils displayed spontaneous release of monomers up to 12 hrs, after which the there was a gradual increasing pattern observed till the end of the study (48 hrs) (Fig. 10A, SST fib). In contrary, the ncSST fibrils showed only a slow release of monomers, with slight increment in Trp fluorescence after 24 hrs (Fig. 10A, ncSST fib). Our results thus suggest that the ncSST fibrils release monomers at a much slower rate compared to the SST fibrils, indicating that the fibrils formed from the non-cyclic SST might be structurally more intact as compared to SST fibrils.

To decipher if the slower monomer releasing tendency possessed by ncSST fibrils compared to SST fibrils is an inherent property of the fibrils, we evaluated the thermal and chemical denaturation profiles of SST and ncSST fibrils. Two methods were employed viz. equilibrium unfolding using guanidine hydrochloride (GdnHCl) and temperature-induced denaturation. Guanidine hydrochloride-mediated denaturation as
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monitored by tryptophan fluorescence showed that SST fibrils got dissociated at low concentrations (0.5 or 1M) of GdnHCl and above these concentrations saturation was reached (Fig. 10B, SST fib). On the other hand, ncSST fibrils showed gradual denaturation with increasing concentration of GdnHCl (Fig. 10B, ncSST fib) indicating resistance to chemical denaturation of these fibrils are relatively higher than that of SST fibrils. Similar studies using GdnHCl have previously been performed to analyze in detail the conformational strength of amyloid fibrils in vitro (56-58).

Temperature-induced denaturation is another method to study the structural stability of proteins and amyloid fibrils (59-62). To evaluate thermal denaturation of SST versus ncSST fibrils, both fibrils were subjected to increasing temperature (25-95°C) and Trp fluorescence of the samples was monitored. For SST fibrils, Trp fluorescence experiments showed considerable red shift of $\lambda_{max}$ (~340 to ~380 nm) at 45°C itself and attained saturation, whereas for ncSST fibrils, only a relatively small $\lambda_{max}$ red shift was ~340 to ~350 nm was observed at this temperature, which almost remained constant thereafter (Fig. 10C). Our thermal denaturation results thus suggest that SST fibrils are thermally more labile compared to ncSST fibrils. Overall, we find the results from chemical/thermal denaturation studies to be consistent with that of the monomer release assay, all of which clearly demonstrate that ncSST fibrils exhibit a relatively higher thermal and GdnHCl resistance, which might influence its monomer releasing potency.

DISCUSSION

Whilst it was previously known that the protein/peptide hormones are stored as aggregates within SGs, the molecular organization was not clearly understood, till recent evidences suggested that these hormones are stored as amyloids within SGs of the endocrine cells (4). The effective utilization of amyloids as a depot for hormone storage/secretion would depend primarily on the reversible nature of aggregates. In other words, the aggregates must encompass the property to store the peptide/proteins in a stable fashion and release monomers when necessary. The aggregation of the stored proteins in SGs has been suggested to be influenced by changes in environment conditions and various modulating factors (38,63-65). Additionally, the native conformation of the proteins/peptides and its dynamicity could also alter its aggregation behavior.

The current study is aimed at understanding the effect of subtle changes in native structure of a peptide hormone and its potential manifestation on the aggregation and release profiles of its aggregate. In order to probe the relationship between the native structure and aggregation of peptide, we chose somatostatin-14, an important peptide hormone that is secreted via regulated secretory pathway (66). The presence of a single disulfide bridge between residue Cys$^3$ and Cys$^{14}$ in somatostatin-14 makes it a simple yet attractive model system to probe altered conformational dynamics and aggregation in response to slight modifications in native structure (S-S bond cleavage). Disulfide bonds have been previously reported to play a key role in modulating the intrinsic dynamics of proteins which are crucial to their native function (10,67,68). In somatostatin and its peptidic analogues, the importance of disulfide bond on its structural and aggregation features has been demonstrated previously (69,70).

In the present work, we compared the cylic and non-cyclic somatostatin for their conformational flexibility, amyloid formation kinetics and fibril reversibility in order to get insights on the role of native ‘cyclic’ structure (-S-S- integrity) relevant to storage and secretion of the hormone. Conformational dynamics controlling aggregation and fibril formation by SST and ncSST-Somatostatin during the course of aggregation was observed to transit multiple stages before forming fibrils (Fig. 3A). However, it was interesting to observe that ncSST displays faster aggregation kinetics compared to SST (Fig. 3A and 4A). One possible explanation for this event could be that the linearization of the peptide possibly exposed the ‘aggregation-prone’ regions for interpeptide interaction allowing instant amyloid aggregation in presence of heparin. Comparing the secondary structure of the SST and
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ncSST in fibrillar state (from CD and FTIR studies), we found SST to display a ‘mixed’ secondary structure, whereas ncSST shows a classical β-sheet (Fig. 3A, G and Fig. 4A, G). We infer from these results that the disulfide bond could affect the secondary structure in SST fibrils.

Next, we investigated whether any differences in conformational dynamics of SST and ncSST could be the underlying factor for observed variation in aggregation kinetics and fibril properties. To answer this question, we first determined the structure of the peptide using solution-state NMR and probed the effect of disulfide bond on its conformational flexibility using all-atom molecular dynamics. The lack of any distinct structure for SST or ncSST is clear from our NMR results. However, upon addition of heparin, SST assumed a structure that was resolvable (Fig. 5E) by NMR and we used this as the starting structure for our MD simulations. The MD simulations reveal that the restriction in backbone flexibility of SST due to the S-S bond causes it to display a two-state dynamics (Fig. 6A). On the other hand, the disulfide reduced structure accesses a wider range of conformations and is highly dynamic (Fig. 6). Disulfide bond has previously been suggested to reduce conformational dynamics, increase mechanical stability and reduce entropy in proteins/peptides (10,67,71). Additionally, this linkage has been shown to play a role in minimizing protein aggregation (14,15,72) thereby making it an effective modulator of aggregation. In this context, disulfide bonds have been suggested to limit the aggregation tendencies of highly aggregation-prone stretches in hIAPP and insulin (73). The findings of the present work imply that the presence of disulfide bond in SST not only plays a crucial role in limiting the conformations accessed by the peptide, but also alters its aggregation profile.

We further studied differences in aggregation of SST and ncSST at an atomistic level using MD simulation. Simulations in the absence of heparin in the solvent box reveal an inherent tendency of somatostatin to form small aggregated clusters, which are short-lived and relatively smaller in size (Fig. S1C). Interestingly, the addition of heparin shifted the dynamics towards formation of larger aggregated clumps composed of more number of monomers. The immediate clustering might be due to the formation of stable interactions between heparin backbone and peptides and reduction of the intrinsic dynamicity of the peptide structure. Addition of heparin may also increase the local concentration of the peptide around heparin that leads the stable interpeptide interactions resulting in higher order aggregates. The 100 ns simulations of both SST and ncSST in the presence of heparin showed a strong tendency of the peptide to self-assemble with formation of intermolecular hydrogen bonds during the course of aggregation (Fig. 7 and 8). However, a detailed analysis of the self-assembled clusters in presence of heparin revealed that the network of stable H-bonds in ncSST was markedly different in comparison to its cyclic counterpart (Fig. 7C and 8C). It is interesting to note that an organized interpeptide H-bond network was observed in ncSST, whereas in case of SST, the H-bond receptors and donors were scattered along the length of the peptide. These differences in H-bonding pattern suggest that upon release of disulfide bond, the hydrogen bonding network shows greater organization in terms of the location of H-bond donors and acceptors along the primary structure.

When we specifically probed for amino acid residue stretch that acts as the amyloid core using H/D exchange (coupled with NMR) and proteinase K digestion experiments, we found that almost the entire peptide participated in the amyloid formation (Fig. 9), further corroborating our in silico findings. Consistent with our observation, the aggregation-prediction algorithm Zyggregator also displayed that residues 3 to 14 in the peptide are aggregation-prone (Fig. 9B). Similar findings, where almost the entire peptide/protein was observed to be structured or protected within the fibrils have been reported for other proteins previously (74,75).

Reversibility and denaturation of SST and ncSST fibrils- For secretory granule biogenesis, the controlled formation of protein aggregates during maturation of SG at trans-Golgi is of
utmost importance (2,76-78). However, it also required that the granules dock onto the plasma membrane and release their contents in a controlled manner upon receiving external signal (2,38,79). Therefore, the peptide/protein hormone amyloids inside the granules require stability for it to be suitably stored in addition to being reversible, allowing it to release monomers to the extracellular space. Dysregulation of release could thus lead to hormone related disorder(s). For instance, it was suggested that R183H mutation in growth hormone results in prolonged retention thus impaired release of this hormone (80), leading to autosomal dominant GH deficiency. However, the role played by the native structure of the protein/peptide in controlling its aggregation and amyloid formation associated with secretory granule biogenesis is not well understood. When we probed for differences in ‘fibril reversibility’ resulting from disrupted disulfide linkage in somatostatin, we found that SST amyloids release the monomers relatively at a faster rate than the ncSST fibrils when exposed to physiological pH (Fig. 10A). This could most likely be due to the difference in structural arrangement of monomers within the respective fibrils. We however cannot negate the possibility of heparin’s role in modulating fibril intactness.

Further, thermal and guanidine hydrochloride-mediated denaturation of fibrils reveal that ncSST amyloids possess relatively higher resistance to denaturing conditions compared to SST amyloids (Fig. 10B and C). This could be due to the greater accessibility to the aggregation-prone state upon release of disulfide bond constraints, different interpeptide H-bonding pattern with increased involvement of hydrophobic residues as well as increased peptide-heparin interactions. For hormones to be released to the extracellular space, the fibrils should readily release the monomers, which might not happen if the naturally occurring fibrils were composed of ncSST. Therefore, SST fibrils must have been selected by nature for regulated storage and secretion, over the ncSST fibrils.

**CONCLUSION**

Peptide/protein hormones have recently been demonstrated to be stored as amyloids within the secretory granules. Storage and secretion of these hormones might be influenced by a multitude of factors including the post-translation modification and native structure of the protein. Here, we attempted to understand whether a change in the native structure (disulfide reduction) of a representative peptide hormone somatostatin-14 leads to any difference in its conformational dynamics, aggregation profile and fibril disassembly in the context of hormone aggregation, storage and release. We propose that the increased conformational flexibility due to linearization (cyclic$\rightarrow$non-cyclic) plays a vital role in modulating not only the kinetics of amyloid formation, but also the nature of resultant aggregates in terms of fibril intactness and reversibility (Fig. 11). Defective disulfide bond formation during post-translational modifications could thus have significant implications related to storage, secretion and function of the peptide hormones.
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FOOTNOTES

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1To whom correspondence should be addressed: Samir K. Maji, Department of Biosciences and Bioengineering, IIT Bombay, Mumbai 400 076, India, Tel: + (91-22) 2576-7774, Fax: + (91-22) 2572 3480, Email: samirmaji@iitb.ac.in, Ashutosh Kumar, Department of Biosciences and Bioengineering, IIT Bombay, Mumbai 400 076, India, Tel: + (91-22) 2576-7762, Email: ashutoshk@iitb.ac.in
6These authors contributed equally to this work
#The abbreviations used are: D-man, D-mannitol; GAGs, glycosaminoglycans; GdnHCl, guanidine hydrochloride; Hep, heparin; ncSST, non-cyclic somatostatin (disulfide reduced); PBST, phosphate buffered saline with Tween 20; SG, secretory granules; SST, somatostatin (native)
4This article contains supplemental figures S1-S2 and a supplemental table S1. Supplemental information file contains additional data from MD simulations and NMR experiments.

FIGURE LEGENDS

FIGURE 1. (A) Primary structure of somatostatin-14 (SST-14) showing disulfide bridge between residues Cys³ and Cys¹⁴. (B) The ball and stick model of somatostatin-14 in its cyclic (left) and non-cyclic (right) forms.

FIGURE 2. Storage state of somatostatin-14 as amyloids within the rat hypothalamus tissue. Photomicrographs of the hypothalamic periventricular nucleus (PeVN) neurons showing somatostatin immunofluorescence visualized using AlexaFluor647-conjugated secondary antibody (red). Note the colocalization of somatostatin (red) with amyloid-specific dye Thioflavin S (green, panel A) or amyloid-specific antibody OC (green, panel B) indicating that SST-14 are stored as amyloids within the secretory granules. Scale bar: 10 μm

FIGURE 3. Somatostatin-14 amyloid formation. (A) Conformational transition of somatostatin-14 in presence of heparin during aggregation measured by CD spectroscopy. (B) ThT fluorescence (at 482 nm) recorded during aggregation of SST. (C) Tryptophan fluorescence of somatostatin-14 in presence of heparin showing blue shift of λmax during aggregation. In the absence of heparin, Trp fluorescence is mostly unaltered. (D) Time-resolved fluorescence intensity decay kinetics of SST monomer Vs fibrils; the smooth lines indicate fits generated to a three-exponential function. (E) Stern-Volmer plots of SST and SST fibrils derived from dynamic quenching of Trp fluorescence using lifetime measurements. N-acetyl tryptophan amide (NATA) was used as standard reference control (F) Electron micrographs showing morphologies of the SST during aggregation. The time-point at which sample was obtained is indicated
Disulfide bond regulates somatostatin-14 amyloid formation

FIGURE 4. Aggregation of non-cyclic somatostatin-14. (A) Conformational transition measured by CD spectroscopy during ncSST aggregation. (B) ThT fluorescence (at 482 nm) recorded during ncSST amyloid formation. (C) Tryptophan fluorescence of ncSST showing blue shift of $\lambda_{\text{max}}$ immediately after addition of heparin, which thereafter remains unaltered. (D) Time-resolved fluorescence intensity decay kinetics of ncSST monomer Vs fibrils; the smooth lines indicate fits generated to a three-exponential function. (E) Stern-Volmer plots of ncSST monomer and ncSST fibrils derived from dynamic quenching of Trp fluorescence using lifetime measurements. N-acetyl tryptophan amide (NATA) was used as standard reference control. (F) Electron micrographs showing the morphological changes at various stages in ncSST aggregation. The time-point at which sample was obtained is indicated (in mins or hrs) on each image shown above; Scale: 200 nm. (G) FTIR spectra of ncSST monomer and ncSST fibrils; the ncSST fibrils showing a peak at $\sim$1633 cm$^{-1}$ indicative of the $\beta$-sheet and another peak at $\sim$1664 cm$^{-1}$ that can be assigned to 3$\alpha$10 Helix. (H) Congo red (CR) staining of ncSST fibrils display apple-green birefringence under cross-polarized light. Left panel indicates the corresponding bright field image. (I) The ncSST fibrils (ncSST fib) displaying the increase in CR absorbance compared to ncSST monomer. Only CR in buffer was used as a control.

FIGURE 5. Structural characterization of SST-14. (A) and (B) showing the 1D $^1$H NMR and TOCSY spectrum respectively, of SST (blue) and SST-Hep (red). (C) Regions of NOESY spectrum showing medium and long-range NOEs, which aided in structure calculation of SST-14. (D) Ensemble of 10 structures of somatostatin (in presence of heparin) calculated using CYANA program. (E) SST-14 average structure from the ensemble showing the N-terminus, C-terminus and amino acids in sequence. (F) 1D $^1$H spectra of ncSST monomer in water (containing 5% D-mannitol) in absence or presence of heparin. (G) and (H) respectively represent the assigned TOCSY and NOESY spectra of ncSST-14. Availability of only sequential peaks in NOESY and secondary chemical indices close to zero (I) clearly shows that the non-cyclic form of SST-14 assumes no definite structure in water.

FIGURE 6. Conformational dynamics of somatostatin monomer. (A) Distribution of the radius of gyration of the various states of the peptide sampled during the 200 ns simulation. SST-14 (blue) shows a predominantly two state behaviour, while ncSST-14 (red) displays increased conformational sampling. (B) The representative structures from the various states accessed by SST-14 (top panel) and ncSST-14 (bottom panel) during the 200 ns simulation arranged in the order of most accessed (extreme left) to least accessed (extreme right). The five most frequented clusters in case of SST-14 were accessed with a frequency of 52% 10%, 4%, 3.2% and 2%, respectively. However, in case of ncSST-14, the most frequented clusters were accessed with lower frequencies of 8.1%, 6%, 4%, 3.2% and 2%, respectively.

FIGURE 7. Characterization SST-14 aggregation using MD simulations. (A) Initial (left) and final
Disulfide bond regulates somatostatin-14 amyloid formation

(right) states of the 24-peptide system in absence (top panel) and presence (bottom panel) of heparin. (B) Interpeptide hydrogen bonds (H-bonds) in the presence and absence of heparin showing significantly higher numbers of H-bonds in presence of heparin in the system. (C) Interpeptide H-bond network in SST; only those bonds stable for >15% of the simulation time were considered. The heat map indicates the number of peptide pairs displaying each H-bonded interaction. (D) Total surface area of the peptides in the simulation box showing a decrease in the presence of heparin while remaining mostly unaltered in the absence of heparin in the simulation box. (E) Radius of gyration of the 24-peptide SST system in presence and absence of heparin showing initial decrease and thereafter unchanged behaviour for SST-Hep during simulation. In absence of heparin, the radius of gyration showed an increasing trend which was fluctuating throughout the simulation.

FIGURE 8. Characterization of ncSST-14 aggregation using MD simulations. (A) Initial (left) and final (right) states of the 24-peptide ncSST system in absence (top panel) and presence (bottom panel) of heparin. (B) Interpeptide hydrogen bonds (H-bonds) in the presence and absence of heparin showing increase in H-bonds with time in presence of heparin while significantly fewer numbers of H-bonds were observed in absence of heparin. (C) Interpeptide H-bond network in ncSST; only those bonds stable for >15% of the simulation time were considered. The heat map indicates the number of peptide pairs displaying each H-bonded interaction. (D) Total surface area of peptides after 100 ns simulation showing lower cumulative surface area in presence of heparin. (E) Radii of gyration showing opposite different trends in ncSST in presence and absence of heparin during simulation.

FIGURE 9. Elucidation of somatostatin fibril core. (A) Proteinase K digestion profiles of fibril (left) and monomer (right) as analyzed by mass spectrometry at different time-points of digestion. The result showing a peak at ~1637 Da corresponding to full-length peptide in the fibril sample suggesting monomer is resistant to enzyme digestion in the fibrillar state (left), however it gets degraded in its monomeric state (right). The masses of the digestion products are labelled adjacent to each peak; peak a and peak b in ‘FIBRIL’ sample representing fragment mass of 1277 Da (SST fragment excluding K9-T10-F11) and 1404 Da (SST fragment excluding F11-T12), respectively. (B) Aggregation-prediction algorithm Zyggregator suggesting almost the entire peptide participates in amyloid formation. (C) H/D exchange coupled with 2D NMR of SST in fibrillar state shown at time 0 (d0) and after day 8 (d8). The overlay (rightmost) of d0 and d8 shows slight alteration in peak intensities of all the residues. (D) Histogram showing the relative variation in peak intensity in individual residues on d0 and d8 of SST fibrils. Most of the peaks showed only slight difference in intensity after 8 days of exchange. (E) Proteinase K digestion profiles of ncSST fibril (left) and monomer (right) as analyzed by mass spectrometry at the mentioned time-points. The fibrils upon proteinase K digestion did not show any fragments up to 480 minutes, whereas the MONOMER being susceptible to proteinase-digestion showing fragmentation by 10 mins. The FIBRIL sample shows a peak at ~1638 (full length SST) throughout the incubation period with proteinase K, suggesting that almost the entire peptide performs as the fibril core. (F) H/D exchange (coupled with 2D NMR) illustrate that most amide protons in the ncSST fibrils (d0, in H2O) remain un-exchanged even after incubation in D2O for 8 days. The rightmost panel shows the overlay of the spectra of d0 and d8. Out of 14 amide protons, three amide protons showing differences in intensity after H/D exchange for 8 days are shown in (G).
FIGURE 10. Monomer release and denaturation profile of SST and ncSST fibrils. (A) Monomer release assay showing that the SST fibrils release monomers at a faster rate compared to ncSST fibrils, at pH 7.4 (relevant to extracellular pH). (B) GdnHCl-mediated denaturation of SST and ncSST fibrils showing changes in \( \lambda_{\text{max}} \) of Trp fluorescence in presence of increasing GdnHCl concentrations. ncSST fibrils showed more resistance to GdnHCl denaturation. (C) Thermal denaturation of SST and ncSST fibrils, showing ncSST fibrils are relatively more resistant to temperature-induced denaturation compared to SST fibrils. The ‘fib’ term indicates fibrils.

FIGURE 11. Native structure controlling aggregation of somatostatin. The native somatostatin (cyclic) is restricted to display a two-state conformational dynamics due to the constraint imposed by the disulfide linkage between Cys\(^3\) and Cys\(^{14}\) residues. The peptide aggregates to form amyloid fibrils upon prolonged incubation and the resulting amyloids give away the monomers instantly. In contrast, the cleavage of disulfide bond in somatostatin leads to increased conformational flexibility, rapid aggregation kinetics resulting in fibrils with increased structural integrity (resistance to denaturation), which release monomers at a slower rate as compared to its native form.
FIGURES

Figure 1

A

\[ \text{AGC}^3 \text{KNFFWKTFTSC}^{14} \]

B

SST-14

ncSST-14
Disulfide bond regulates somatostatin-14 amyloid formation

Figure 2

A  SST  ThioS  Merge

B  SST  OC  Merge
Disulfide bond regulates somatostatin-14 amyloid formation
Disulfide bond regulates somatostatin-14 amyloid formation

Figure 4
Figure 6

A

\[
P(R_g) \\
\text{vs. } R_g (\text{Å})
\]

B

1 2 3 4 5

SST

ncSST
Disulfide bond regulates somatostatin-14 amyloid formation

Figure 7

A

SST

0 ns

100 ns

SST-Hep

0 ns

100 ns

B

Interpeptide

--- SST
--- SST-Hep

No. of H-bonds

Time (ns)

0

20

40

60

80

100

C

SST (Hep)

H-Bond Donor

H-Bond Acceptor

D

Surface Area

--- SST-Hep
--- SST

Surface Area (nm)

Time (ns)

0

50

100

E

Radius of gyration

--- SST-Hep
--- SST

Rg (Å)

Time (ns)

0

50

100
Figure 8

A

ncSST

0 ns

100 ns

ncSST-Hep

0 ns

100 ns

B

Interpeptide

No. of H-bonds

0 20 40 60 80 100

Time (ns)

C

ncSST (Hep)

H-Bond Donor

H-Bond Acceptor

D

Surface Area

Surface Area (nm)

0 50 100

Time (ns)

E

Radius of gyration

Rg (Å)

0 24 40

Time (ns)
Figure 9

Disulfide bond regulates somatostatin-14 amyloid formation
Figure 10

A. Trp fluorescence (normalized) vs. time (hrs)

B. Trp fluorescence vs. GdnHCl [M]

C. Trp fluorescence vs. temperature [°C]
Figure 11

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