Doctoral Thesis

Physical stability of peptide pharmaceuticals aggregation and conformational changes of human calcitonin (hCT) in aqueous solution

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Physical Stability of Peptide Pharmaceuticals: Aggregation and Conformational Changes of Human Calcitonin (hCT) in Aqueous Solution

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Abstract

Serious difficulties in the formulation and delivery of peptide and protein drugs are rooted in their inherent tendency to undergo various instability processes. Consequently, for rational stabilization strategies, the physical and chemical properties of such molecules have to be understood at the molecular level. Due to the complexity of protein conformations and behaviour, their analysis must encompass a wide range of methods. These methods should assess the protein's properties in such a way that changes are identified which have the potential to affect stability, function, safety and efficacy.

For developing a new drug delivery system for the peptide hormone human calcitonin (hCT), highly concentrated (i.e. in the millimolar range), chemically and physically stable solutions were required. Conflicting with this approach is the fact that in aqueous solution hCT has the tendency to aggregate, resulting in viscous and/or gelatinous and often turbid dispersions.

The turbidity kinetics of aqueous hCT solutions was measured spectrophotometrically at 350 nm. The time course of turbidity revealed important qualitative information. The aggregation appeared to be driven by a nucleated condensation mechanism as the time course of the aggregation kinetics consisted of a lag phase, followed by a sigmoidal rise of turbidity and ending in a plateau. The aggregation process was enhanced by increasing the hCT concentration, temperature, ionic strength and the pH of the solution in the acidic solution range. The shortening of the lag phase when increasing ionic strength and pH towards the isoelectric point may be explained by a critical reduction of the electrostatic repulsion between hCT monomers. The nucleation theory was strengthened by the fact that the hCT aggregation of freshly prepared hCT solutions could be seeded by addition of small volumes of pre-aggregated hCT samples.

To understand the aggregation process of hCT in more detail, the dominating interparticle interactions were investigated by static and dynamic laser light scattering experiments. The assumption of a pair interaction potential consisting of a hard core, a screened electrostatic and a van der Waals term, together with concepts from the theory of complex fluids yielded a self-consistent description of the experimental data. This allowed to calculate a stability diagram, which predicts the physical stability of hCT solutions as a function of ionic strength and hCT concentration. However, we were not successful in monitoring the kinetics of hCT aggregation by laser light scattering as this technique observes only a
very small sample volume. Thus, the randomly distributed formation of critical nuclei could not be detected. Additionally, severe problems arose from the apparent minute concentrations of such nuclei combined with the fast growth of large hCT aggregates, which precipitated rather quickly after nucleus formation, hindering the accurate monitoring of very small particles such as critical nuclei.

Fluorescence spectroscopy was performed to monitor the aggregation phenomena of hCT using both the intrinsic fluorescence of hCT and the fluorescence properties of the hydrophobic probe Nile red adhering to hCT. Of special interest was the lag phase before turbidity became apparent. Alterations in fluorescence properties of Nile red bound to hCT, i.e. increase in relative intensity, blue shift in emission maximum, and changes in its polarization degree, provided information both on incipient steps and on final states of the hCT aggregation process which were inaccessible by turbidity measurements. In contrast to the other methods used, i.e. measurements of turbidity and of intrinsic fluorescence, the Nile red fluorescence technique revealed that the maturation of fibrillar hCT assemblies is a slow process.

Attenuated total reflection Fourier transform infrared (ATR-FTIR) spectroscopy was used to monitor in situ the adsorption of hCT from a bulk solution onto a hydrophobic/hydrophilic interface. The adsorption process at this interface was accompanied by partial formation of an amphiphilic α-helix. The initial adsorption became then superimposed by aggregation phenomena characterized by significant β-pleated sheet formation. Because aggregation of hCT is associated with conformational changes, the secondary structure sensitive amide I'-band (D2O) could be used as a diagnostic marker for this aggregation process.

Using various electron microscopical techniques, namely scanning (SEM), transmission (TEM), and scanning transmission electron microscopy (STEM), a characteristic polymorphism of the fibrillar hCT assemblies was observed. It is proposed that after nucleation hCT aggregation starts with the formation of approx. 4 nm thick protofibrils. Such protofibrils may further interact via lateral association and coiling to form higher ordered fibrillar assemblies, i.e. protofibril-ribbons, fibrils, fibril-ribbons, tubes, and cables. The transitions involved between the various fibrillar hCT assemblies represent a maturation process as previously indicated in the investigations using Nile red fluorescence.

Based on data from various analytical techniques, a model for the conformational changes of hCT during aggregation and an outline of the supramolecular arrangement of hCT within polymorphic fibrillar aggregates is proposed. In conclusion, the detailed insight in the hCT aggregation mechanism may contribute to the development of stable, highly concentrated aqueous formulations of hCT, and
furthermore, is very likely to be useful for the understanding of aggregation phenomena of other peptide and protein drugs.
Zusammenfassung


Für neuartige, alternative Applikationsformen von humanem Calcitonin (hCT) werden hochkonzentrierte Lösungen im millimolaren Konzentrationsbereich benötigt, die chemisch und physikalisch hinreichend stabil sind. Diesem Ziel steht die Tatsache entgegen, dass hCT in wässrigen Lösungen zur Aggregation neigt. Die Aggregation führt zu viskosen und/oder gelartigen und oft trüben Dispersionen.


Ein tieferes Verständnis des Aggregationsprozesses konnte durch eine Untersuchung der dominierenden interpartikulären Wechselwirkungen mittels statischer und dynamischer Laser-Streulichtexperimente erreicht werden. Unter Annahme eines Paarwechselwirkungspotentials bestehend aus einem 'hard core',

Mit Hilfe von Fluoreszenz-Spektroskopie wurden die Aggregationsphänomene von hCT verfolgt. Dabei benutzte man sowohl die intrinsische Fluoreszenz des hCT als auch die Fluoreszenz-Eigenschaften des hydrophoben Farbstoffes Nilrot (NR), der sich in wässrigen Lösungen an hCT-Moleküle anhaftet. Von speziellem Interesse war die lag-Phase bevor die ersten Anzeichen einer Trübung auftreten. Änderungen der Fluoreszenz-Eigenschaften des an hCT-gebundenen NR, d.h. Zunahme der relativen Intensität, Verschiebung des Emissionsmaximums nach kleineren Wellenlängen und Änderungen des Polarisationswertes, lieferten sowohl Informationen über Anfangs- als auch über Endstufen des hCT-Aggregationsprozesses. Solche Informationen waren aus den Trübungsmessungen nicht zu erhalten. Im Gegensatz zu den anderen angewandten Methoden (Messung der Trübung und der intrinsischen Fluoreszenz) konnte mit der NR-Fluoreszenzmethode ein sehr langsam ablaufender, langanhaltender Reifungsprozess der fibrillären hCT-Aggregate nachgewiesen werden.

Mit Fourier-Transformations-Infrarot-Spektroskopie unter Bedingungen der abgeschwächten Totalreflexion (ATR-FTIR) konnte die Adsorption von hCT-Molekülen aus der Bulklösung an eine hydrophobe/hydrophile Grenzfläche unter in situ Bedingungen untersucht werden. Der Adsorptionsprozess an der Grenzfläche war begleitet von einer partiellen Bildung einer amphiphilen α-Helix. Die anfängliche Adsorption wurde anschliessend überlagert durch den Aggregationsprozess, der durch die signifikante Bildung eines β-Faltblattes charakterisiert ist. Da die Aggregation von hCT mit Konformationsänderungen verknüpft ist, kann die auf Sekundärstruktur-Änderungen reagierende Amid I'-Bande (D₂O) als Charakteristikum für die Aggregation benutzt werden.

Mit verschiedenen elektronenmikroskopischen Techniken, nämlich Raster-, Transmissions- und Raster-Transmissions-Elektronenmikroskopie konnte ein
interessanter Polymorphismus der hCT-Aggregate aufgedeckt werden. Gemäß der EM-Untersuchungen beginnt nach der Nukleation die hCT-Aggregation mit der Bildung von ca. 4 nm dicken Protofibrillen. Protofibrillen können durch seitliches Aneinanderlagern und Zusammendrehen zu höher geordneten Aggregaten weiter reagieren, nämlich zu Protofibrillen-Bändern, Fibrillen, Fibrillen-Bändern, Röhren und Kabeln. Die Übergänge zwischen den verschiedenen fibrillären hCT-Aggregaten stellen einen langsam ablaufenden Reifungsprozess dar, der sich schon bei den Fluoreszenzstudien mit NR angedeutet hatte.

Die durch die verschiedenen Untersuchungen gewonnenen Erkenntnisse erlauben es, ein Modell für die Konformationsänderungen des hCT während der Aggregation und für die Anordnung der hCT-Moleküle innerhalb der polymorphen fibrillären Aggregate aufzustellen. Der detaillierte Einblick in den hCT-Aggregationsmechanismus kann einerseits zu der Entwicklung stabiler, hochkonzentrierter wässriger hCT-Formulierungen beitragen und andererseits hilfreich sein für das Verständnis von Aggregations-Phänomenen anderer Peptid- und Proteinarzneistoffe.