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A comparative study of structure, stability and function of sc-tenecteplase in the presence of stabilizing osmolytes

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ABSTRACT

The aim of the present study was to investigate the effect of three routine drug excipients, as osmolytes, in three different concentrations, on structure, thermal stability and the activity of single-chain (sc-) tenecteplase. To see the influence of trehalose, mannitol, and sucrose on the structure, stability and function of sc-tenecteplase, thermal stability, fluorescence, circular dichroism (CD) and enzyme kinetic measurements and molecular docking studies were carried out. To measure the effect of osmolytes on stability of sc-tenecteplase, thermodynamic parameters (transition temperature (T_m), standard enthalpy change (ΔH°), standard entropy change (ΔS°) and ΔG°, the standard Gibbs free energy change, were determined from heat-induced transition curves of the protein in absence and presence of each osmolyte. It was observed that all three osmolytes acted as an enhancer for the sc-tenecteplase stability, with varying efficacies and efficiencies. The results of the kinetic study showed that the activity of sc-tenecteplase is increased in the presence of osmolytes. The near-UV and far-UV CD studies showed transfer of Trp, Phe and Tyr residues to a more flexible environment in the presence of osmolytes. The sc-tenecteplase fluorescence quenching suggested the more polar location of Trp residues. Molecular docking studies revealed that (i) Gibbs free energy of interaction between the osmolyte and sc-tenecteplase is negative, and (ii) hydrogen bond and hydrophobic interactions dominate within the interaction sites.

1. Introduction

Changing environmental conditions impose a great deal of stress on most organisms. To adapt such variable harsh conditions, living organisms produce and accumulate certain small organic molecules, known as osmolytes. It is well known that stabilizing osmolytes not only stabilize proteins and induce folding of aberrant proteins, but also their presence in the cell does not largely alter protein functional activity (Arakawa and Timasheff, 1985; Bolen, 2004; Singh et al., 2011; Taneja and Ahmad, 1994; Yancey et al., 1982). Besides, promising implications on biotechnology, health and industry make the osmolyte-protein science a potential research area for many researchers, even those working in different fields of science. One potential field is to take advantage of such beneficial molecules as excipients of drug formulations in order to confer stability in solution and even during freezing of biopharmaceuticals (Singh et al., 2011; Taneja and Ahmad, 1994).

The solvent-induced stabilization of proteins is specific and related to the excipients in the formulation, however, it is achieved by strengthening of the protein-stabilizing forces, by destabilization of the denatured state, or by direct binding of excipients to the protein (Wang, 1999). Besides, the structure of water surrounding the folded protein is of great importance in maintaining the structure of the protein, that's why excipients are typically added to replace missing interactions (i.e., resulting from drying) or to increase the interactions (i.e., to stabilize) (Mattos and Clark, 2008). On the other hand, in the presence of a stabilizing excipient, the protein is preferentially hydrated (or the excipient is preferentially excluded), that is, more water molecules are found on the surface of the protein than in the bulk, and such a process is believed to stabilize the protein (Arakawa et al., 2001; Timasheff, 2002; Wang, 1999).

Drug proteins and peptides are mostly formulated as injectables, and because of the poor stability of proteins, the processing of the pharmaceutical product does not allow for terminal sterilization. Instead, a sterile filtration step is usually included in the production and intensive control of the active substance and excipients are necessary, namely a low bioburden and absence of endotoxins (Jorgensen et al., 2009; Shukla and Thömmes, 2010).

Proteins are frequently more stable in the solid state, so formulations of powder for reconstitution are often chosen to increase stability. Freeze-drying (lyophilization) and spray-drying are two frequently used

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drying methods (Arakawa et al., 2001; Cleland et al., 1993; Tang and Pikal, 2004); common for both processes of the removal of water (solvent) from the liquid formulation. Stabilizing excipients are often added to stabilize proteins during the stress induced by a freeze-drying process (Jorgensen et al., 2009; Shukla and Thömmes, 2010).

A freeze-dried cake needs to have a certain appearance to be useful and it should be rapidly dissolvable and blow-out of the formulation must be prevented (Jorgensen et al., 2009; Shukla and Thömmes, 2010). Excipients added to optimize a dry powder formulation are called bulking agents. Sugars (i.e., trehalose and sucrose) and polyols (i.e., mannitol) are the stabilizing excipients that can have cryoprotectant and/or lyoprotectant effects on the protein (Kett et al., 2004; Kumar et al., 2009; Wang, 2000) during both the freezing and the drying processes. Besides, they are routine bulking agents (Anhorn et al., 2008).

There are other criteria for choosing pharmaceutical excipients including availability from a reliable supplier and being obtainable in the right quality with a controlled level of residual solvents, preferably of non-animal origin. Moreover, excipients must be produced according to GMP requirements, therefore, the ones that are controlled by pharmacopoeial monographs are often preferred. In this way, well-known excipients (e.g., polyols and sugars) that have been recognized and used for a long time are preferable (Jorgensen et al., 2009; Shukla and Thömmes, 2010).

Tenecteplase is a fibrin-specific, triple-combination mutant of human t-PA (tissue plasminogen activator) produced in a Chinese hamster ovary cell line by recombinant DNA technology, developed to circumvent some of the limitations of current thrombolytic therapy (Parsons et al., 2012). Human t-PA was first isolated as a single-chain serine proteinase with MW of about 70 000, consisting of 527 amino acids and 17 disulfide bonds with Ser as the N-terminal amino acid. The t-PA molecule contains 5 domains: finger domain, epidermal growth factor-like domain, two kringle domains and a serine proteinase region with the active-site residues His322, Asp371, and Ser478 (Collen and Lijnen, 2009).

Tenecteplase has some pharmacokinetic advantages over t-PA. It provides a balance between efficacy and risk of bleeding in the treatment of stroke at lower dose than the dose used for acute myocardial infarction (AMI) (Tanswell et al., 2002). Other characteristics of this valuable biotherapeutic protein, which highlights the importance of research on it are: significantly enhanced safety profile, a 5-s, single-bolus injection, a longer half-life, increased resistance to plasminogen activator inhibitor, and improved fibrin specificity compared with t-PA (Haley et al., 2005; Semba et al., 2001).

The wealth of information on mechanistic and potential applications of osmolytes in pharmaceutical industry must be aptly utilized by researchers working in translational and clinical aspects to gear up for use as therapeutic for many human diseases. Thus, understanding the cause of protein instability or aggregation should aid in the design of an appropriate formulation (Jorgensen et al., 2009; Shahid et al., 2017; Shukla and Thömmes, 2010).

The aim of the present investigation was to study the effects of three osmolytes, namely mannitol, sucrose and trehalose (three routine excipients) at three concentrations, on thermal denaturation and structure of sc-tenecteplase. Tenecteplase is a valuable therapeutic protein which might be easily available in one-chain as well as two-chain forms, the research on which have the potential of providing novel and different insight on osmolyte influence on protein structure. The interaction of the above-mentioned small molecules was investigated by UV–vis and fluorescence spectroscopy as well as circular dichroism.

2. Materials and methods

2.1. Materials

Metalyse, recombinant, single chain-tenecteplase (sc-TNK), was obtained from Boehringer Ingelheim. Mannitol, trehalose, sucrose, phosphoric acid, L-arginine, tissue plasminogen activator substrate (T2943), monobasic and dibasic sodium phosphates, and Tris-base were purchased from Sigma. Tween-20 was procured from Merck Co.

Solutions were made in double-distilled water. Buffer (pH 7.2) contained 0.04% tween-20, 300 mM L-arginine and 173 mM phosphoric acid, which is similar to tenecteplase formulation as a biotherapeutic protein. Trehalose, sucrose and mannitol were added to the buffer to the final concentrations of 50, 100 and 200 mM. To remove salts, present in the protein, the reconstituted sc-tenecteplase was diafiltered in Amicon Ultra-0.5 ml Centrifugal Filters for several times. Buffers were filtered using a 0.2 µm Millipore filter paper, and the concentration of the protein was determined using Eppendorf BioPhotometer D30 (Eppendorf AG, Germany) and a molar absorption coefficient of 109,250 M^{-1} cm^{-1}. All experiments were done in the L-arginine containing buffer except circular dichroism (CD) measurements, which were done in 50 mM sodium phosphate buffer containing 0.1% polysorbate-20 (pH 7.2).

2.2. Methods

2.2.1. Thermal denaturation study

Thermal denaturation studies were carried out in Cary 100 Bio UV–vis Spectrophotometer (Agilent Technologies, United States), with a heating rate of 1.0 °C/min. Each sample was heated from 25 to 85 °C. Change in absorbance with increasing temperature was followed at 280 nm. The basic observation for each protein was a heat-induced transition curve, i.e., a plot of an optical property against temperature. Buffer ingredients, namely tween-20 and arginine, ensure protein aggregation inhibition (Carpenter et al., 2002) which affects the absorption.

2.2.2. Circular dichroism measurements

The CD spectra were obtained using a spectropolarimeter model AVIV 215 (Aviv Biomedical Inc., United States). The buffer, osmolytes, and sc-tenecteplase were pre-equilibrated at room temperature for 15 min. The secondary structure of sc-tenecteplase was monitored by the far-UV CD (190–260 nm); sc-tenecteplase concentration was 0.2 mg/ml (3.3 µM), and 0.1 cm path length cuvette was used. The change in tertiary structure of the protein was measured by the near-UV CD (260–350 nm); sc-tenecteplase concentration was 0.5 mg/ml (8.3 µM), and 1.0 cm path length cuvette was used. Each spectrum was recorded twice and averaged. The observed ellipticity at wavelength λ (θ_λ), measured in millidegrees, subtracted from buffer and expressed as molar ellipticity, $[\theta]_\lambda$, in degree cm^2/mol using the relation, $[\theta]_\lambda = \theta_\lambda / (10 \times c \times l)$ where c is the molar concentration of the sample (mole/l), and l is the path length in cm. Since L-arginine interferes in far-CD measurements, the L-arginine containing buffer was substituted with 50 mM sodium phosphate buffer (pH 7.2), while the amount of polysorbate-20 increased to 0.1% to counteract low solubility of the protein in the absence of L-arginine (Tischer et al., 2010).

2.2.3. Fluorescence spectra measurements

Intrinsic fluorescence emission spectra of tenecteplase were recorded in the wavelength range 295–440 nm, after exciting the protein at 280 nm at 298 K. All samples were prepared, and their concentration were adjusted to 0.1 mg/ml (1.6 µM) in various concentrations of mannitol, sucrose and trehalose, equilibrated for 10 min. An Agilent Cary Eclipse Fluorescence Spectrophotometer (Agilent Technologies, United States) was used to record the intrinsic fluorescence of the protein. The slit widths were 5 and 10 nm for excitation and emission scans, respectively.

2.2.4. UV absorption spectra measurements

To see tertiary structure changes in the presence of various concentrations of the three osmolytes, Cary 100 Bio spectrophotometer