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Research paper

The effect of GlycoPEGylation on the physical stability of human rFVIIa with increasing calcium chloride concentration

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ABSTRACT

The effects of calcium chloride on the structural, kinetic and thermal stability of recombinant human factor VIIa (rFVIIa) were investigated using rFVIIa and two GlycoPEGylated recombinant human FVIIa derivatives, a linear 10 kDa PEG and a branched 40 kDa PEG, respectively. Three different $CaCl_2$ concentrations were used: 10 mM, 35 mM and 100 mM. The secondary structure and tertiary structure of rFVIIa at 25 °C, measured by circular dichroism (CD), were maintained upon GlycoPEGylation as well as $CaCl_2$ content. In contrast, the thermal stability of the three rFVIIa compounds, measured by differential scanning calorimetry (DSC) and circular dichroism (CD), and aggregation behaviour, measured by light scattering (LS), were affected by the increasing calcium concentration. Increasing the $CaCl_2$ concentration from 10 mM to 35 mM resulted in a decrease in the apparent unfolding temperature, T_m, of rFVIIa, whereas the concentration of $CaCl_2$ has to be raised to 100 mM in order to see the same effect on the GlycoPEGylated rFVIIa compounds. The temperature of aggregation of rFVIIa, T_{agg}, increased as the $CaCl_2$ concentration increased from 35 mM to 100 mM, while T_{agg} for the GlycoPEGylated rFVIIa compounds was practically independent of the $CaCl_2$ concentration. From the obtained results, it is concluded that GlycoPEGylation postpones the calcium induced thermal destabilisation of rFVIIa, and a much higher calcium concentration also postpones the thermally induced aggregation of rFVIIa. The thermally induced aggregation of the GlycoPEGylated rFVIIa compounds is unaffected by an increasing calcium chloride concentration.

1. Introduction

For blood coagulation factor VIIa (FVIIa), the presence of calcium ions is vital, as these ions assist in initiating the blood coagulation cascade including the association of tissue factor (TF), and following the activation of factor IX and factor X resulting in a burst of thrombin, fibrin deposition and the formation of a haemostatic plug on the platelet surface [1]. Deficiencies in the coagulation system due to partial or complete deficiency of FVIII or FIX, haemophilia A or haemophilia B, respectively, can lead to severe morbidity or mortality if the bleeding is left untreated.

A safe and efficient way to prevent bleeds and joint destruction in haemophilia is prophylactically by dosing factor (F) FVIII or FIX 2–4 times weekly [2]. Recent studies have shown that prophylactic treatment with recombinant FVIIa (rFVIIa, NovoSeven®) in haemophilia patients with inhibitors against FVIII or FVIX reduces the frequency of bleedings significantly when compared to conventional on-demand haemostatic therapy [3]. However, based on the half-life and circulation time of rFVIIa (2–4 h), it is assumed that rFVIIa should be administered daily if used for long-term prevention [3,4]. Hence, development of rFVIIa derivatives with longer circulation time could result in both fewer administrations and better patient compliance. Modification of pharmaceutical proteins with hydrophilic polymers, such as poly-ethylene–glycol (PEGylation), is an established method for prolonging circulatory half-life, reducing self-aggregation, increasing water solubility and increasing stability [5,6]. Due to the risk of losing activity of FVIIa because of the numerous interactions with the cell surface, TF, FIX and FX, there is a limitation in the unspecific chemical modification of this protein. For this reason, a novel strategy for site-directed PEGylation using glycosyltransferases to attach PEG to glycan residues, the enzyme based GlycoPEGylation™ technology, is used to covalently attach either a linear 10 kDa or a branched 40 kDa PEG polymer to rFVIIa, a technique originally described by DeFrees et al. [7]. The site of PEG attachment to rFVIIa is demonstrated to be one of the two N-linked glycans of rFVIIa (Asn145 or Asn322) located on the light chain and heavy chain, respectively [8]. It is assumed that the specific location of the GlycoPEGylation site and presence of small amounts of di-GlycoPEGylated species are irrelevant in relation to the overall interpretation of the obtained results.

From a pharmaceutical formulation point of view, altering the salt concentration in the protein buffer is of interest in relation

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to its effect on the protein stability. Addition of electrolytes to proteins in solution can have complex effects on the physical stability of the protein. Electrolytes can modify the conformational stability, the equilibrium solubility (e.g. salting-in and salting-out) and the formation of aggregates [9]. Salts can interact with the unpaired charged side chains of proteins and stabilise or destabilise the native state of the protein. The former is a result of the binding of mainly multivalent ions to the unpaired charged surface available side chains, which can lead to cross-linking between the charges and thus stabilisation. The latter is found, e.g., if the ions bind more strongly to the unfolded state of the protein than to the native state [9]. A number of physical properties of aqueous salt solutions follow the rank order of the Hofmeister series, which arrange solutes according to their ability to act as kosmotrope (salting-out) or chaotrope (salting-in) agents [10,11].

In this study, the effect of $CaCl_2$ on the thermal, kinetic and structural stability of rFVIIa and GlycoPEGylated rFVIIa compounds is investigated. Ca^{2+} binding is an important stabiliser for a number of proteins, and structural aspects of the specific Ca^{2+} binding to rFVIIa are of great importance for understanding its physiological function [12]. There are a total of 9 specific binding sites for Ca^{2+} in three distinct regions on rFVIIa, and they all have different affinities [13,14]. The binding of these 9 Ca^{2+} ions are vital for the initiation of the blood coagulation cascade, as the binding of Ca^{2+} is a prerequisite for the high-affinity binding of rFVIIa to TF [14–16]. All the Ca^{2+} binding sites are saturated when the calcium buffer concentration exceeds 10 mM [13]. In the present study, we have used three $CaCl_2$ concentrations ranging from 10 mM to 100 mM to make sure all the specific binding sites of rFVIIa are saturated and hence focus the study on the effect of low affinity binding of Ca^{2+}. The physical stability of liquid protein drugs is an important property to investigate as insoluble aggregates may increase immunogenic responses and are not biologically active. $CaCl_2$ is a potential salting-in agent, which interacts more strongly with the unfolded form than with the native form, consequently shifting the equilibrium towards the unfolding reaction [11]. This behaviour is pronounced at concentrations around 100 mM [11,17]. Investigating the stability of rFVIIa and GlycoPEGylated rFVIIa in higher concentrations of $CaCl_2$ than 10 mM is of relevance in relation to the pharmaceutical development, purification and formulation processes, during which the protein concentration often exceeds 1 mg/mL (1 mg/mL NovoSeven® contains 10 mM $CaCl_2$). When concentrating rFVIIa, an addition of $CaCl_2$ is needed in order to keep rFVIIa in solution. In contrast, the absence of visual precipitate suggests that GlycoPEGylated rFVIIa stays in solution in 10 mM $CaCl_2$ even at high concentration (>10 mg/mL).

The effect on the physical stability rFVIIa of increasing the $CaCl_2$ concentration is investigated by using rFVIIa, and two GlycoPEGylated® rFVIIa compounds, a 10 kDa linear kDa and 40 kDa branched PEG. This provides us with a relevant pharmaceutical model system for studying the effect of an increasing Ca^{2+} concentration on both a native protein and its two GlycoPEGylated compounds. Biophysical characterisation tools, including circular dichroism (CD), differential scanning calorimetry (DSC) and light scattering (LS), were used to study possible changes in the secondary and tertiary structure as well as the thermal and kinetic stability of rFVIIa, 10 kDa PEG-rFVIIa and 40 kDa rFVIIa upon an increase in $CaCl_2$ concentration.

2. Materials and methods

2.1. Materials

rFVIIa, 10 kDa GlycoPEGylated rFVIIa and 40 kDa GlycoPEGylated rFVIIa were produced by Novo Nordisk A/S, Denmark., as described in [8]. L-Histidine was purchased from Aijonomoto AminoScience (Raleigh, N.C.), calcium chloride dihydrate from Merck (Germany), Barium Chloride 20 w/w from Ampliqon (Denmark), 0.1 N iodine solution from Sigma–Aldrich (Germany), 70% perchloric acid from Merck (Germany), LDS sample buffer and MES SDS running buffer and Simple Blue Safe Stain all from Invitrogen (Carlsbad, CA). All protein solutions were dialysed in Slide-A-Lyzer™ 30.000 MWCO dialyse cassettes against the desired buffer. Three different buffers were used, only varying in $CaCl_2$ content. Buffer 1 contained 10 mM histidine (HIS) and 10 mM $CaCl_2$. Buffer 2 contained 10 mM HIS and 35 mM $CaCl_2$. Buffer 3 contained 10 mM HIS and 100 mM $CaCl_2$. All buffers were adjusted to pH 5.75.

2.2. SDS–PAGE

SDS–PAGE analysis was carried out using a 12% Bis-Tris gel from Invitrogen. The gels were loaded with an average of 5 μg protein per well and run at 120 mA constant current. The running buffer was MES running buffer. The gel was washed in 150 mL 0.1 M perchloric acid for 15 min until 40 mL 5% barium chloride solution and 15 mL 0.1 M iodine solution were added to detect protein bands containing PEG compounds, as described in [18]. After discolouring in water, the gel was coloured with Coomassie blue.

2.3. MALDI-TOF MS

Mass spectrometric analysis was performed on a Bruker Daltonics Microflex MALDI-TOF (Billerica, MA) equipped with a nitrogen laser (337 nm). The instrument was operated in linear mode with delayed extraction, and the accelerating voltage in the ion source was 25 kV. Then, 1 μL sample solution was mixed with 10 μL matrix solution (alphacyano-cinnamic acid dissolved in a 5:4:1 mixture of acetonitrile:water:3% TFA), and 1 μL of this mixture was deposited on the sample plate and allowed to dry before insertion into the mass spectrometer. Calibration was performed using external standards (a range of standard proteins), and the resulting accuracy of the mass determinations is within 0.1%.

2.4. Circular dichroism (CD) spectroscopy

Spectra of rFVIIa in the far-UV region (200–260 nm) were recorded on a Jasco J-810 CD Spectropolarimeter (JASCO International Co. Ltd., Hachioji City, Japan). The light path of the cuvette was 0.05 mm, and a protein concentration of 2.4 mg/mL was used. The spectra were recorded at room temperature. Each spectrum is an average of five scans. A value of 114 g/mol was used as a mean residue weight for rFVIIa. Spectra of rFVIIa in the near-UV region (250–350 nm) were recorded on the same Spectropolarimeter. The light path of the cuvette was 10 mm, and a protein concentration of 2.4 mg/mL was used. The spectra were recorded at room temperature. Spectra at selected temperatures in the range of 10–80 °C were obtained in a 2-mm cuvette. Each spectrum is an average of five scans. All spectra were background corrected, smoothed and transformed into molar ellipticity (θ cm^2 $dmol^{-1}$) as described in [19].

2.5. Differential scanning calorimetry (DSC)

DSC experiments were performed with a MicroCal VP-DSC (Northhampton, MA). Prior to scanning, all solutions were degassed by stirring under vacuum. A pressure of 2 atm was applied over the cells during scanning, and a scan rate of 1 °C/min was used. The concentration of rFVIIa was 2–2.4 mg/mL, and buffer scans were subtracted from protein scans. DSC data were analysed using the Origin software from MicroCal Inc., supplied with the instrument. A baseline was subtracted prior to analysis. The

apparent denaturation temperature (T_m) values were determined as the temperature corresponding to the maximum C_p, and the errors are determined by repeatable experiments. The enthalpy is calculated from the area under the peak bound by the baseline, and the errors are based on the adjustment of this baseline.

2.6. Light scattering (LS)

The LS experiments were performed with a Wyatt DynaPro Titan (Santa Barbara, CA) that employs a 829-nm laser and collects scattering intensity data at a fixed angle of 90 °C. Cuvette temperature is controlled using a thermoelectric solid-state heating module (Peltier heat pump). Solutions are examined in a quartz cuvette with 12 μL cell volume containing glass viewing windows for in situ scattering measurements. The concentration of rFVIIa was 1–2 mg/mL, and samples were centrifuged at 10.000 RPM for 10 min prior to analysis. A scan rate of 1 °C/min was used.

3. Results

3.1. Purity

After dialysis, the purity of the protein solutions was determined by SDS–PAGE and by MALDI-TOF MS as described and published in [20]. Briefly, it was shown that the rFVIIa sample was pure and contained only rFVIIa with a molecular mass of 50 kDa. The 10 kDa PEG-rFVIIa solution contains a mono-GlycoPEGylated, 60 kDa, and smaller amounts of a di-GlycoPEGylated compound, at 70 kDa, of 10 kDa PEG-rFVIIa. The 40 kDa PEG-rFVIIa solution contains mono-GlycoPEGylated 40 kDa PEG-rFVIIa with a molecular mass of 92 kDa. It is assumed that the specific location of the GlycoPEGylation site and the presence of small amounts of di-GlycoPEGylated species are irrelevant in relation to the overall interpretation of the results obtained in this study.

3.2. Structural characteristics

The secondary structure of rFVIIa and GlycoPEGylated rFVIIa is investigated by far-UV CD, and the results have been reported previously [20]. From that study, it was evident that the far-UV CD scans of rFVIIa and 40 kDa PEG-rFVIIa are inseparable, and there is only a small variation in the far-UV signal at very low wavelength between the native rFVIIa and the 40 kDa PEG-rFVIIa compared to the 10 kDa GlycoPEGylated rFVIIa compound. The near-UV CD scans show no change in the tertiary structure of rFVIIa upon GlycoPEGylation. Due to the high absorbance of Cl^- ions below 200 nm [19], it was not possible to measure far-UV CD scans on samples containing 35 mM and 100 mM $CaCl_2$.

3.3. Thermal, kinetic and structural stability

The effect of increasing the $CaCl_2$ buffer content on the thermal, kinetic and structural stability of rFVIIa and GlycoPEGylated rFVIIa is investigated by three different complementary techniques: DSC, LS and CD. From the CD scans, it is evident that there is no difference between the thermally induced structural changes upon an increase in $CaCl_2$ in rFVIIa and GlycoPEGylated rFVIIa, respectively (Fig. 2).

We chose to follow one characteristic wavelength, 286 nm, throughout the CD heating scan. This wavelength was chosen as the CD signal at this specific wavelength changes markedly upon heating, see Fig. 3. The CD signal at 286 nm reflects primarily the aromatic amino acids, tryptophan (290–305 nm) and tyrosine (275–282 nm) [19].

It is shown in Fig. 3 that from 60 °C and above, the change in the 286 nm CD signal is independent of the $CaCl_2$ content, but different from the unmodified rFVIIa to the GlycoPEGylated rFVIIa compounds. For rFVIIa, there is a clear drop in the CD signal around T_m, whereupon the signal increases and stabilises around 0 deg cm^2 mol^{-1}. On the other hand, the GlycoPEGylated rFVIIa compounds demonstrate a more continuous course. Differentiating the CD signal with respect to the temperature gives an estimate of the unfolding temperature based on the thermally induced structural changes, see Table 1.

The DSC thermograms and the LS intensity data are plotted in the same Fig. 4.

The effect of $CaCl_2$ on the thermal stability of rFVIIa, 10 kDa PEG-rFVIIa and 40 kDa PEG-rFVIIa is investigated by DSC. From the resulting thermograms, it is evident that the thermal stability of rFVIIa depends on both $CaCl_2$ content and GlycoPEGylation (Fig. 4). The apparent unfolding temperature of rFVIIa decreases at 35 mM and 100 mM $CaCl_2$ compared to 10 mM $CaCl_2$, whereas 10 kDa PEG-rFVIIa and 40 kDa PEG-rFVIIa are not thermally destabilised until the $CaCl_2$ concentration reaches 100 mM. There is a substantial difference between the appearances of the thermograms after T_m has been reached. From the DSC thermograms and the LS intensities for the GlycoPEGylated rFVIIa compounds (Fig. 4), it appears that GlycoPEGylation delays the thermally induced aggregation of rFVIIa at 10 mM $CaCl_2$ and 35 mM $CaCl_2$. At 100 mM $CaCl_2$, the unmodified rFVIIa and the GlycoPEGylated rFVIIa compounds display similar aggregation behaviour.

Table 1
Compilation of the thermodynamic and thermal stability data for rFVIIa and GlycoPEGylated rFVIIa.

	T_m[a] (°C) (CD)	T_{onset} (°C) (DSC)	T_m (°C) (DSC)	ΔH (T_m) (kJ/mol)	T_{agg} (°C) (LS)	$T_{onset} - T_{agg}$
10 mM $CaCl_2$						
rFVIIa	60.3 ± 0.2	50.9 ± 0.2	57.7 ± 0.2	NA	50.8 ± 0.4	0.1
10 kDa PEG-rFVIIa	59.9 ± 0.2	53.3 ± 0.1	59.0 ± 0.2	140	57.1 ± 0.2	−3.8
40 kDa PEG-rFVIIa	60.7 ± 0.2	52.9 ± 0.1	59.0 ± 0.2	134	57.9 ± 0.9	−5
35 mM $CaCl_2$						
rFVIIa	59.9 ± 0.2	49.0 ± 0.1	55.2 ± 0.2	NA	49.8 ± 0.7	−0.1
10 kDa PEG-rFVIIa	59.3 ± 0.2	51.0 ± 0.1	58.0 ± 0.2	140	56.8 ± 0.3	−5.8
40 kDa PEG-rFVIIa	59.3 ± 0.2	52.3 ± 0.1	58.6 ± 0.2	153	57.7 ± 0.2	−5.4
100 mM $CaCl_2$						
rFVIIa	61.5 ± 0.2	48.3 ± 0.1	55.1 ± 0.2	NA	59.3 ± 0.4	−11
10 kDa PEG-rFVIIa	59.9 ± 0.2	47.5 ± 0.2	55.3 ± 0.2	147	59.2 ± 0.4	−11.6
40 kDa PEG-rFVIIa	59.7 ± 0.2	47.1 ± 0.1	55.0 ± 0.2	156	56.7 ± 0.2	−9.6

The apparent unfolding temperature, T_m, measured by CD, the onset temperature T_{onset}, the T_m and corresponding enthalpy measured by DSC, the aggregation temperature, T_{agg}, measured by LS and the difference between T_{onset} and T_{agg}. The extraction of the enthalpy of unfolding of rFVIIa is not possible due to the irreversibility of the denaturation process. The DSC and LS experiments were made in duplicates and were reproducible. The errors are based on this experimental reproducibility.

[a] Based on the CD scans in Figs. 1 and 3 as well as [20]. The uncertainties are calculated based on the resolution of the original CD scan.

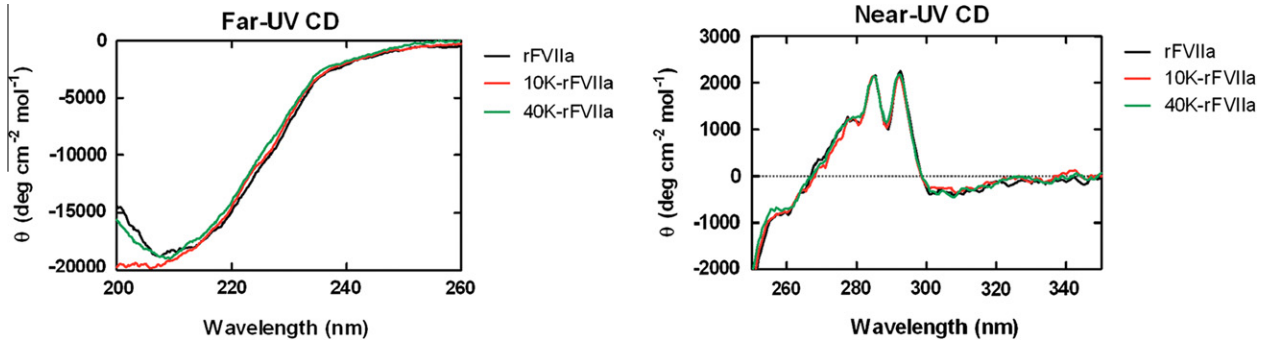

Fig. 1. Far-UV CD scan and near-UV CD scan of rFVIIa, 10 kDa PEG-rFVIIa and 40 kDa PEG-rFVIIa in 10 mM HIS, 10 mM $CaCl_2$, pH 5.75. The far-UV CD scans show only little variation between the spectrum of 10 kDa PEG-rFVIIa and the two spectrums of rFVIIa and 40 kDa PEG-rFVIIa, and the near-UV CD scans show that GlycoPEGylation has no notable effect on the tertiary structure (near-UV CD) of rFVIIa. Results adapted from [20]. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

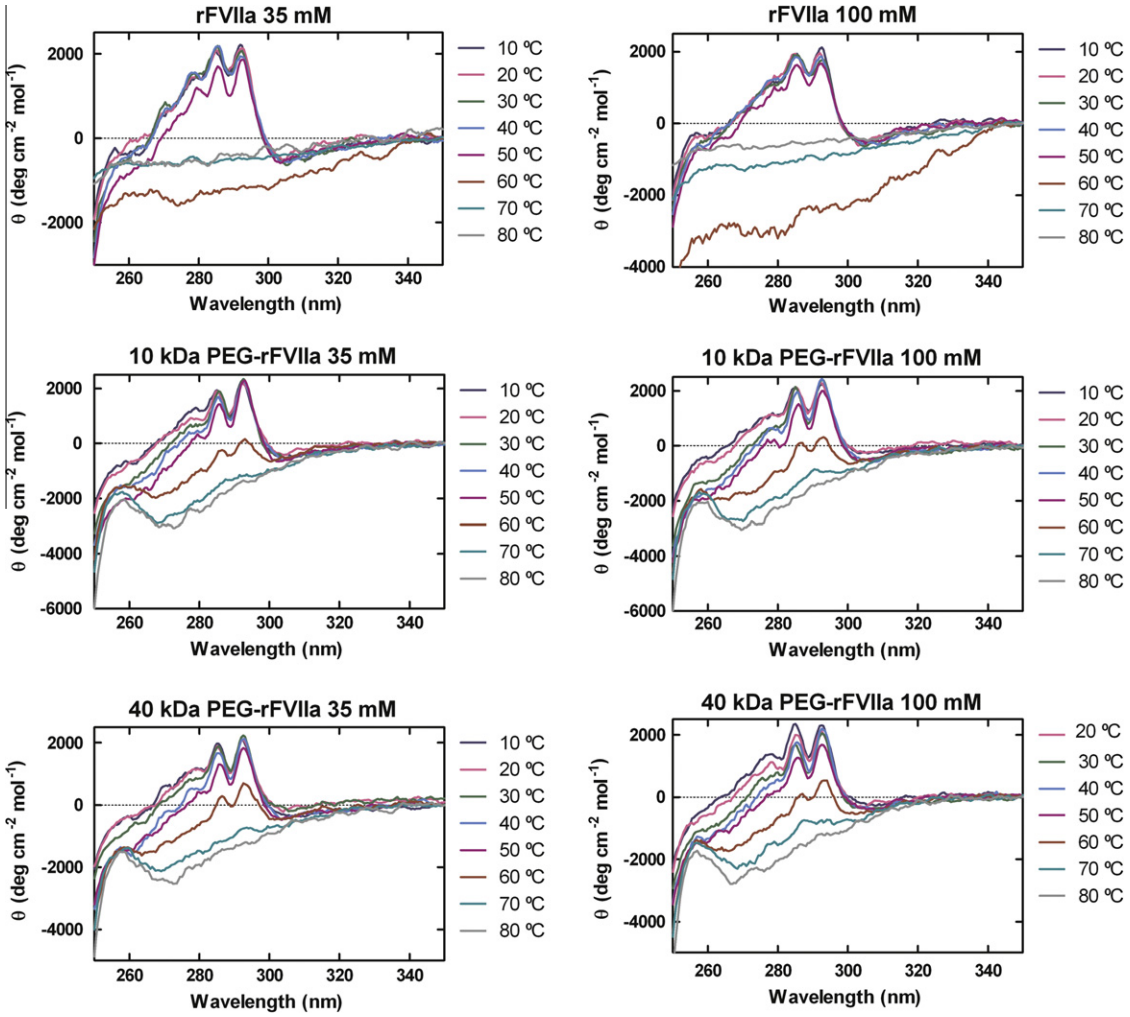

Fig. 2. Near-UV CD at different temperatures (see legends) of rFVIIa, 10 kDa PEG-rFVIIa and 40 kDa PEG-rFVIIa. The left panels show results obtained in 10 mM HIS, 35 mM $CaCl_2$, pH 5.75, and the right panels show results obtained in 10 mM HIS, 100 mM $CaCl_2$, pH 5.75. The spectra in both panels are similar, indicating that there are no or very small differences in the thermally induced unfolding due to increasing $CaCl_2$ content. Both panels also show spectra which indicate that the thermally induced unfolding process differs from the non-modified protein to the GlycoPEGylated proteins. The GlycoPEGylated proteins maintain some residual tertiary structure at 60 °C and above, whereas CD signal from the native protein shows no structural characteristics at these temperatures. The native rFVIIa solution was milk-white upon heating, whereas the GlycoPEGylated rFVIIa compounds were transparent. Similar results are found for rFVIIa and its GlycoPEGylated compounds in 10 mM HIS, 10 mM $CaCl_2$, pH 5.75 [20]. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

4. Discussion

The effect of $CaCl_2$ on the thermal, structural and kinetic stability of rFVIIa was measured by three complementary techniques: DSC, CD and LS. The three different measurement types illustrate each different aspect of the unfolding or aggregation processes. The unfolding and aggregation pathway can be illustrated by a model put forward by Lumry and Eyring in 1954 [21]. According

to the Lumry Eyring model, irreversible protein denaturation involves at least two steps. The first step is the reversible unfolding of the native protein (N), characterised by the rate constants, k_1 and k_2. This step is followed by an irreversible change of the unfolded protein (D) into an inactivated, irreversible aggregate (A), a step characterised by the rate constant k_3. The unfolded state is characterised by having some non-native residual tertiary structure.

$$N \underset{k_2}{\overset{k_1}{\leftrightarrow}} D \xrightarrow{k_3} A, \quad K = \frac{k_1}{k_2}$$

It is our experimental approximation that the aggregation is measured by LS; the unfolding is measured by CD, whereas DSC measures unfolding, an endothermic event, as well as aggregation, an exothermic event. We hypothesize that during the heating scan, there is an equilibrium between the native (N) and the unfolded (D) state, which shifts towards unfolding as the temperature increases. This process is followed by the irreversible step from unfolding (D) to aggregation (A), which is also temperature driven. Our experimental results show that an increasing $CaCl_2$ influences at least two of the three rate constants mentioned above.

The thermally induced unfolding of rFVIIa and GlycoPEGylated rFVIIa at three different $CaCl_2$ concentrations is measured by CD (Fig. 2) and is further illustrated by the changes in the CD signal at 286 nm (Fig. 3). The CD heating scans show that the non-modified rFVIIa undergoes a conformational change in the tertiary structure around the apparent unfolding temperature, and no tertiary structure can be detected at higher temperature. This trend is observed at all calcium concentrations. As the physical appearance of the rFVIIa solution upon heating was milk-white, the non-detectable tertiary structure above 70 °C could be a sign of temperature induced aggregation. The GlycoPEGylated rFVIIa compounds, on the other hand, seem to maintain some non-native residual tertiary structure upon unfolding as the CD signal stabilises around 5000θ cm^{-2} mol^{-1} (Fig. 3).

From the DSC and LS results obtained in 10 mM and 35 mM $CaCl_2$ shown in Fig. 4, it is evident that both the apparent unfolding temperature, T_m, and the temperature of aggregation T_{agg} of the GlycoPEGylated rFVIIa compounds are higher than that of the unmodified protein. There is no notable difference between the 10 kDa GlycoPEGylated rFVIIa and the 40 kDa GlycoPEGylated rFVIIa. The increase in both T_m and T_{agg} with PEGylation is in accordance with previous studies [22–24]. The combined DSC and LS results in Fig. 4 illustrate that the thermally induced aggregation event for rFVIIa begins while endothermic processes dominate. The formation of aggregates will contribute exothermally to the total heat signal. As the heat measured by DSC is the total contribution from both unfolding and aggregation, an extensive formation of aggregates beginning shortly after T_{onset} may shift the location of the peak in the thermogram towards a lower temperature. Increasing the $CaCl_2$ concentration from 10 mM to 35 mM results in a minor decrease in both T_m (measured by DSC) and T_{agg} for rFVIIa, most pronounced is the decrease in T_m. Thus, increasing the $CaCl_2$ concentration appears to result in a decrease in the thermal stability of rFVIIa.

At 100 mM $CaCl_2$, the apparent unfolding temperature measured by DSC is similar for rFVIIa, 10 kDa GlycoPEGylated rFVIIa and 40 kDa GlycoPEGylated rFVIIa. The thermal stability of the GlycoPEGylated rFVIIa compounds increases compared to thermal stability of rFVIIa, when the $CaCl_2$ concentration is increased from 10 mM to 35 mM. The GlycoPEGylated compounds are not thermally destabilised until the concentration of $CaCl_2$ reaches 100 mM. In addition, the thermally induced aggregation of all the investigated compounds appears to occur at a higher temperature at 100 mM $CaCl_2$ compared to the lower calcium concentrations.

It is noticeable how both the native rFVIIa and the GlycoPEGylated rFVIIa compounds display different aggregation behaviours

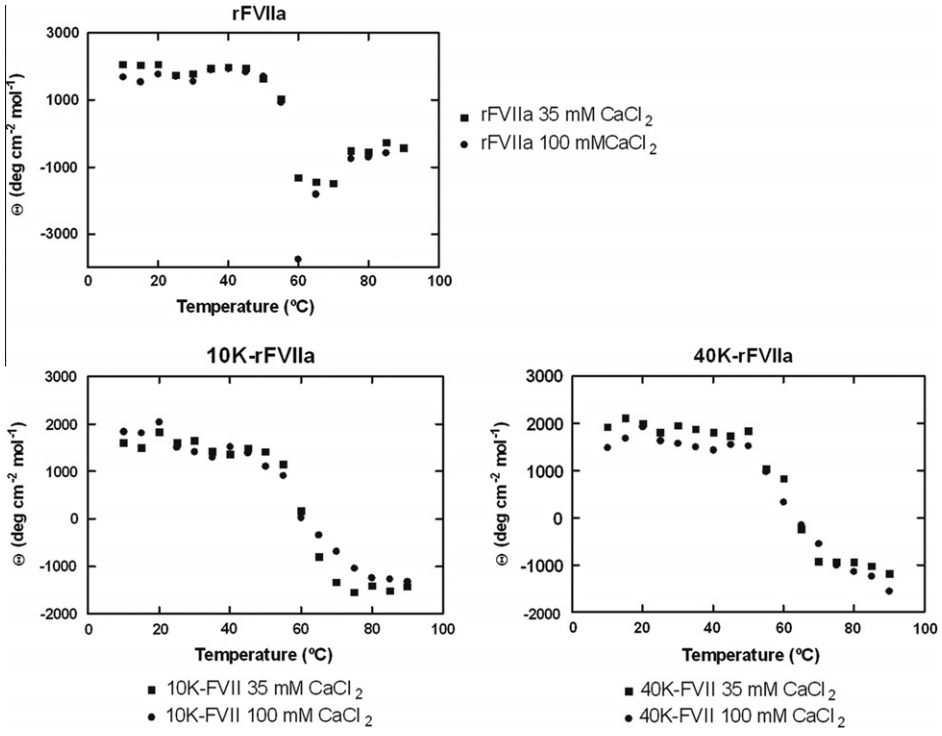

Fig. 3. Near-UV CD signals of rFVIIa, 10 kDa PEG-rFVIIa and 40 kDa PEG-rFVIIa versus temperature at 286 nm in 10 mM HIS, 35 mM $CaCl_2$, pH 5.75 and 10 mM HIS, 100 mM $CaCl_2$, pH 5.75. The temperature dependent near-UV CD signals are independent of the $CaCl_2$ concentration, but different from the unmodified protein to the GlycoPEGylated proteins. The thermally induced unfolding of the two GlycoPEGylated proteins is alike. Differentiating the CD signal with respect to the temperature gives an estimate of the unfolding temperature, see Table 1. Similar results are obtained in 10 mM HIS, 10 mM $CaCl_2$, pH 5.75 [20].

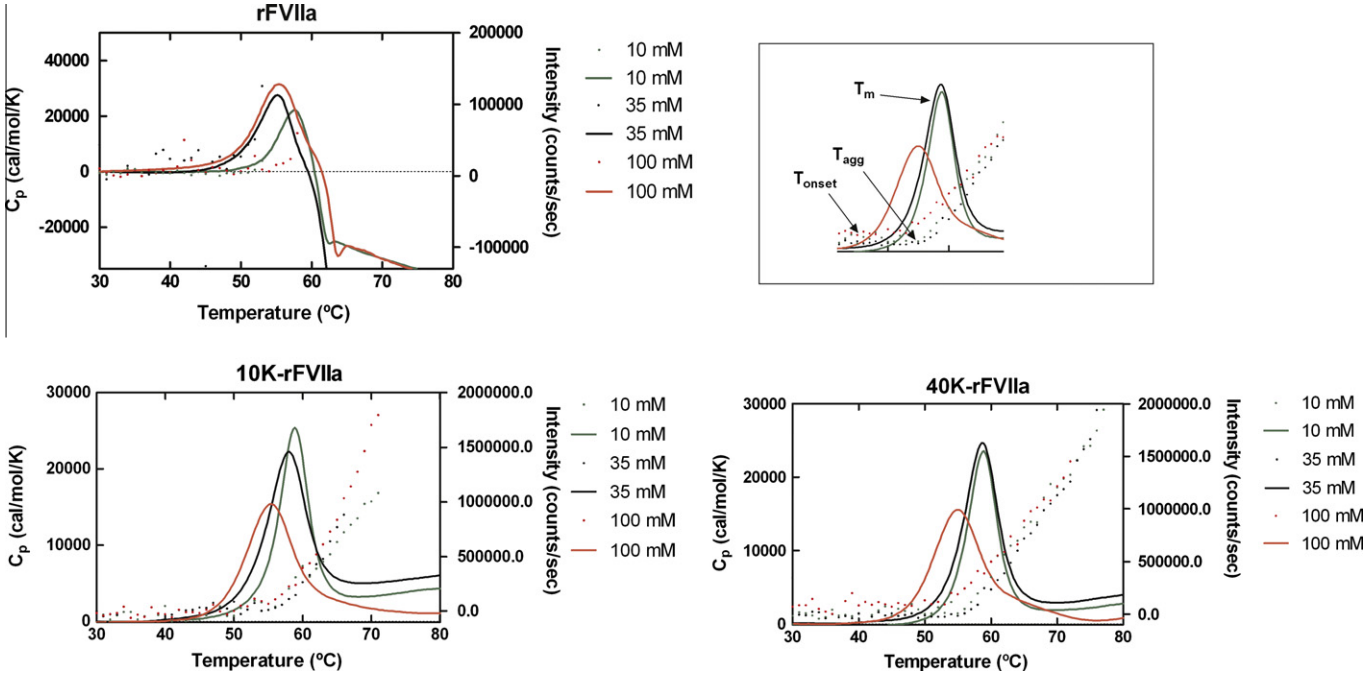

Fig. 4. DSC thermograms and LS intensity measurements of rFVIIa, 10 kDa PEG-rFVIIa and 40 kDa PEG-rFVIIa as a function of temperature in buffers with three different $CaCl_2$ concentrations. The dotted line corresponds to the LS intensity, and the solid line corresponds to the DSC thermogram. The insert illustrates where the onset temperature, T_m, the temperature of aggregation, T_{agg}, and the apparent unfolding temperature, T_m, are found. The rFVIIa aggregates formed close to T_{agg} were too large for the LS detector to measure, and continuous intensity measurements as a function of temperature were impossible after 53 °C for rFVIIa. The obtained results at 10 mM HIS, 10 mM $CaCl_2$, pH 5.75 are from [20]. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

at 100 mM $CaCl_2$. At 100 mM $CaCl_2$, the thermally induced aggregation does not only set in at a higher temperature for all the investigate proteins, native and GlycoPEGylated, the difference between T_{onset} and T_{agg} is 10–11 degrees as opposed to 1–5 degrees at lower $CaCl_2$ concentrations. Based on the LS and DSC measurements, it is suggested that rFVIIa (unmodified and GlycoPEGylated) in general is stabilised by electrostatic interactions that are weakened by the addition of $CaCl_2$ and that $CaCl_2$ might interact preferentially with the protein in the unfolded state, 'D'. Both effects stabilise the aqueous 'D' state, as the equilibrium constant K increases and the rate constant k_3 decreases with increasing calcium concentration, leading to a lower apparent T_m and a higher T_{agg}.

Based on the results obtained in this study, it seems reasonable to assume that the equilibrium constant K, reflecting the equilibrium between the native and the unfolded protein, is independent of the increase in $CaCl_2$ content from 10 mM to 35 mM as well as independent of GlycoPEGylation, since unfolding process for rFVIIa appears to be similar, whether the protein is GlycoPEGylated or not (Fig. 3). The rate constant k_3, on the other hand, describing the step from unfolding to aggregation, decreases dramatically when the protein is GlycoPEGylated. And k_3 of rFVIIa is markedly decreased as the $CaCl_2$ concentration is increased from 35 mM to 100 mM, where T_{agg} increases from ~50 °C at 10 mM and 35 mM $CaCl_2$ to ~59 °C at 100 mM $CaCl_2$. From the LS results in Fig. 4, it is evident that the formation of aggregates is affected by GlycoPEGylation as well as $CaCl_2$ content. At 10 mM and 35 mM $CaCl_2$, the thermally induced rFVIIa aggregates formed close to T_{agg} were too large for the LS detector to measure, and continuous intensity measurements as a function of temperature were impossible. As the light scattering increases with the sizes of the formed aggregates [25], a high intensity count corresponds to large aggregates. The increase in the LS intensity is much slower for the GlycoPEGylated rFVIIa compounds in general; thus, the formation of thermally induced aggregates is reduced and happens at a lower rate. At 100 mM $CaCl_2$, the formation of thermally induced rFVIIa aggregates seems to follow a similar pattern as the GlycoPEGylated rFVIIa compounds. The aggregates are formed slower and to a lower extent compared to the aggregates formed at lower $CaCl_2$ concentration.

5. Conclusion

Recombinant human factor VIIa was GlycoPEGylated with two different PEG polymers, a 10 kDa linear PEG and a 40 kDa branched PEG, and the stability of these compounds was investigated at three different $CaCl_2$ concentrations. The addition of salt, $CaCl_2$, seems to have the same effect on the stability of rFVIIa, regardless of GlycoPEGylation. However, the thermal destabilising effect and postponed aggregation appear to depend on the concentration of $CaCl_2$ once the protein is GlycoPEGylated. The thermal and kinetic effects of increasing the $CaCl_2$ concentration are found at 35 mM for rFVIIa, whereas the concentration of $CaCl_2$ has to be raised to 100 mM in order to see the same effect on the GlycoPEGylated rFVIIa compounds. Thus, GlycoPEGylation postpones the calcium induced thermal destabilisation of rFVIIa, and a sufficiently high calcium concentration also postpones the aggregation of rFVIIa.

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