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The Effect of Shear on the Structural Conformation of rhGH and IgG1 in Free Solution

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ABSTRACT

The effect of hydrodynamic forces on proteins in free solution, also referred to as shear stress in multiple drug substance and drug product processing steps, was investigated by means of *in situ* and inline biophysical measurements. The use of a quartz Couette cell in combination with a circular dichroism spectrometer allowed simultaneously the creation of simple shear flow and direct measurements of the proteins' secondary and tertiary structure. Recombinant human growth hormone and an IgG1 mAb were chosen as model proteins. Under the exclusion of interfacial effects by the addition of a surfactant, no unfolding was observed due to shearing for 30 min up to the highest possible shear rate under laminar flow (3840 s^{-1}). In another experiment, guanidine hydrochloride was added to a surfactant-protected and sheared sample to lower the thermodynamic and mechanical stability of the proteins. However, even under these destabilizing conditions, the proteins showed no change in their secondary and tertiary structure. We conclude that shear stress in terms of velocity gradients is unlikely to unfold the investigated proteins in free solution up to shear rates of at least 10^4 s^{-1}.

Introduction

Since the approval of the first biopharmaceutical in 1982, the market has been growing enormously.[1,2] However, the era of protein-based drugs has brought some new challenges for the pharmaceutical industry. Protein stability over the various processing steps during manufacturing and over the claimed shelf life is a major concern. Besides changes of the primary peptide structure, including deamidation, isomerization, hydrolysis, and oxidation, which are characterized by the formation or cleavage of covalent bonds, protein-based drugs may also suffer from instabilities related to the physical state of chemically unaltered proteins.[3] As a consequence, proteins tend to denature or aggregate, thereby increasing the potential for immunogenicity, changed biological activity, or other adverse effects of the drug.[4]

One of the factors being frequently reported to have an influence on the physical stability of the proteins is shear stress. Looking at the downstream process sequence in the production of biopharmaceuticals, shear can occur in purification, formulation, and fill and finishing steps.[5,6] Many conflicting articles have been published investigating the effect of shear on proteins in solution. A critical review is given by Thomas and Geer.[7] Here we want to provide a deeper insight into the physical mechanism by which shear can harm or otherwise influence protein molecules.

No consensus has been found so far on the effect of shear flow on proteins and the magnitude that is required to trigger unfolding of their structure. Different shear rates are described for a variety of proteins that are required for changes of the secondary and higher structure levels. Beginning at shear rates $<10^2$ s^{-1},[8] fibril formation of amyloid-β,[9] loss of activity of urease[10] and catalase,[11] and dissociation of the tertiary structure configuration of BSA[12] are reported. Conversely, Thomas and Dunnill[13] exposed urease in a capillary at shear rates up to 10^6 s^{-1} but did not find any evidence of deactivation. In a more recent study by Bee et al.,[14] IgG1 was sheared in a parallel plate and a capillary rheometer, reaching shear rates up to 2.5×10^5 s^1. However, even at these high rates, the authors could not observe deleterious effects.

Another key aspect for understanding is the exact definition of shear stress. Biddlecombe et al.[15,16] considered shear stress as a

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combination of hydrodynamic forces and interfacial effects. Using a rotating disk, they created a high shear environment at solid–liquid interfaces and observed protein aggregation over rotational time. Desorption of unfolded proteins from solid–liquid interfaces by hydrodynamic forces was proposed as underlying mechanism for particle creation in a later study by Perevozchikova et al.[17] However, so far, most articles focus on velocity gradients in free solution that excludes—at least theoretically in the evaluation and interpretation of the data—any interfacial effects.

The basic idea behind stress on proteins in terms of velocity gradients is a decrease in the free energy of unfolding (ΔG_U) in free solution itself. By lowering the thermodynamic stability of the protein, velocity gradients could cause a potential shift in the equilibrium toward partially unfolded protein.[18] Many studies performed offline analytics and correlated enzyme inactivation or aggregation and particle creation over time with partial unfolding due to shearing.[8] However, a shift in thermodynamic equilibrium of a protein that shows rapid folding/unfolding kinetics and refolding can only be visualized by *in situ* measurements when the overall fold of the proteins is determined inline during shearing.[18]

A few *in situ* studies have been conducted that investigated a potential partial unfolding of proteins under shear. Jaspe and Hagen[18] studied horse cytochrome c (12 kDa) in a silica capillary. The fluorescence of the protein, which would increase sharply on unfolding, was determined while forcing the sample through the capillary. No evidence of destabilization could be found by the authors at shear rates up to 10^6 s^{-1}. However, they predicted that the required shear rate for unfolding decreases with the molecular weight of the protein. Controversial observations were made by Bekard et al. who used a Couette cell for the creation of simple shear flow. Secondary and tertiary structures of BSA (66 kDa) and bovine insulin (6 kDa) were visualized by far-UV circular dichroism (CD) and fluorescence spectroscopy, respectively.[12,19] The *in situ* measurements revealed a time-dependent and irreversible change in secondary and tertiary structure of the proteins already at low shear rates. Interfacial effects, such as desorption from solid–liquid interfaces, were not part of the discussion.

(Partial) Unfolding of proteins simply by velocity gradients in free solution as it is described by Bekard et al. would have a major impact on the product quality in biopharmaceutical manufacturing steps. Therefore, we conducted a study on our own using a similar setup to Bekard et al. consisting of a Couette cell in combination with a CD spectrometer that allowed *in situ* and real-time structure determinations in the far- and near-UV range. The study was designed to focus primarily on the effect of velocity gradients on proteins in free solution without the influence of interfacial effects. The minimization of interfacial effects was a key parameter in the experimental setup. It enables to draw a conclusion on whether protein denaturation by hydrodynamic forces can only occur at interfaces or also in free solution. Recombinant human growth hormone (rhGH) and an IgG1 mAb were selected as therapeutic model proteins that are members of pharmaceutically highly relevant groups of proteins. These proteins strongly differ in the native protein fold and molecular size, thereby covering a broad range of properties, structural parameters, and molecular masses (22-145 kDa). Besides further investigation of the effect of hydrodynamic forces in terms of velocity gradients on the native protein fold, we additionally studied artificially weakened molecular structures. Lowering the mechanical[20] and thermodynamic stability of proteins by addition of the denaturant guanidine hydrochloride (GuHCl) allowed simulating the effect of very high hydrodynamic forces on the structural stability of the proteins.

Materials and Methods

Materials

rhGH and an IgG1 mAb were provided by Sandoz GmbH (Kundl, Austria) in form of frozen stock solutions of 10.1 and 20.8 mg mL^{-1} (rhGH) and 29.7 mg mL^{-1} (IgG1) in concentration. Sodium phosphate was obtained from Merck (Darmstadt, Germany), citric acid from Sigma–Aldrich (St. Louis, MO), Poloxamer 188 from BASF (Ludwigshafen, Germany), and Polysorbate 80 from J.T. Baker (Center Valley, PA). The isoelectric point of rhGH and IgG1 is pH 5.0-5.1 and pH 9.5-9.9, respectively. RhGH has a molecular weight of about 22 kDa and IgG1 of about 145 kDa.

Methods

Preparation of Samples

The stock solution of rhGH was formulated in 10 mM phosphate (pH 7.0). Dilutions were performed using formulation buffer with and without Poloxamer 188. The antibody was buffered in 25 mM citrate (pH 6.5). Samples were diluted adding formulation buffer with and without Polysorbate 80.

Determination of Melting Temperature

The melting temperatures of the proteins in surfactant-containing and surfactant-free solutions were determined from wavelength transitions in the far-UV and near-UV range and by intrinsic fluorescence spectroscopy. For preparation of surfactant-containing samples 0.03 mg mL^{-1}, Poloxamer 188 was added to the rhGH solution and 0.047 mg mL^{-1} PS80 was added to the IgG1 solution. CD transitions were measured with a Chirascan plus spectrometer (Applied Photophysics, Leatherhead, UK) while ramping the temperature at a rate of 1°C min^{-1} from 70°C to 95°C (far-UV) or 60°C to 90°C (near-UV). Transitions of rhGH were determined in triplicate at a fixed wavelength of 209 and 296 nm and a bandwidth of 1 nm, by averaging the signal over a period of 4 s. The measurements were carried out in 1-mm quartz cuvettes at protein concentrations of 0.8 mg mL^{-1} (far-UV) and 9.9 mg mL^{-1} (near-UV). The melting temperature of the IgG1 was determined by far-UV CD and by intrinsic fluorescence spectroscopy, the latter with an LS55 fluorescence spectrometer (PerkinElmer, Waltham, MA). IgG1 transitions in the far-UV at a protein concentration of 0.5 mg mL^{-1} were determined in triplicate at a fixed wavelength of 222 nm and a bandwidth of 1 nm using a 0.5-mm cuvette and averaging the signal over 4 s. Fluorescence was excited at a wavelength of 295 nm with a slit of 5 nm, and emission was recorded from 320 to 370 nm with a slit of 4 nm at medium gain and a protein concentration of 0.1 mg mL^{-1}.

Determination of GuHCl-Dependent Unfolding Transitions

Far-UV and near-UV spectra of solutions at the indicated GuHCl concentrations in 0.5-mm quartz cuvettes were recorded at 20°C using the Chirascan CD spectrometer. CD data were collected from 205-260 and 250-350 nm every 0.5 nm with a response time of 0.5 s and a bandwidth of 1 nm. Far-UV data of GuHCl-free samples were recorded from 180 to 260 nm. All spectra were baseline normalized, and the spectra were corrected to 0 mdeg at 260 nm.

Unfolding was performed at room temperature in formulation buffer containing the indicated concentrations of GuHCl. A 7.9 M GuHCl stock solution (Sigma–Aldrich) was used for preparation of the denaturation buffers. For determination of far-UV, rhGH was diluted to 0.152 mg mL^{-1} and the IgG1 to 1 mg mL^{-1}. Near-UV denaturation curves were recorded at a concentration of 2.5 mg mL^{-1} (rhGH) and 10 mg mL^{-1} (IgG1); 0.03 mg mL^{-1} Poloxamer 188 was added to the rhGH solutions and 0.047 mg mL^{-1} PS80 was