Oxidation, aggregation and immunogenicity of therapeutic proteins
Torosantucci, R.

Citation
Torosantucci, R. (2013, September 17). *Oxidation, aggregation and immunogenicity of therapeutic proteins*. Retrieved from https://hdl.handle.net/1887/21762

Cover Page

The handle http://hdl.handle.net/1887/21762 holds various files of this Leiden University dissertation.

Author: Torosantucci, Riccardo
Title: Oxidation, aggregation and immunogenicity of therapeutic proteins
Issue Date: 2013-09-17

Chapter 9

Summary and perspectives

Summary

Recombinant human protein therapeutics have unique structural and biological features which make them useful to treat several diseases [1].

However, a drawback of this class of drugs is that they are immunogenic, i.e. a proportion of patients develop anti-drug antibodies (ADAs) during treatment [2]. Among the many factors involved in protein immunogenicity, protein aggregation has been identified as an important risk factor [3]. Aggregates that are generated through oxidative stress have been found to be particularly immunogenic [4-7]. The aim of this thesis was to investigate the mechanisms behind protein aggregation as a result of metal catalyzed oxidation (MCO) [8], and to study the relationship between oxidation, aggregation and immunogenicity of therapeutic proteins.

Chapter 2 is a literature review of the structural and pharmaceutical consequences of oxidation observed in protein and peptide therapeutics, focusing on antibodies, calcitonin, granulocyte colony-stimulating factor, growth hormone, interferon alpha and beta, insulin, oxytocin and parathyroid hormone.

Chapter 3 deals with the aggregation behavior under stress conditions of PEGylated insulin compared with its unpegylated counterpart. Recombinant human insulin was conjugated on lysine B29 with 5-kDa PEG and subjected to heating at 75 °C, metal-catalyzed oxidation, and glutaraldehyde cross-linking. Under each of the applied stress conditions, insulin and PEGylated insulin showed comparable degradation profiles. All the stressed samples were shown to contain submicron aggregates in the size range between 50 and 500 nm. Several stressed samples also contained micron sized particles. Mainly covalent aggregates composed of protein molecules with different degrees of modification in the secondary and tertiary structure were measured, depending on the stress method applied. PEGylation, however, was shown not to affect the sensitivity of insulin towards aggregation [9].

Therapeutic proteins such as recombinant human interferon alfa, recombinant human interferon beta and monoclonal antibodies form highly immunogenic

aggregates induced via copper catalyzed oxidation. In **Chapter 4** the chemical mechanism responsible for insulin aggregation induced by MCO with Cu^{2+}/ascorbate, is described. Oxidized insulin was shown to contain DOPA (3,4-dihydroxyphenylalanine) and DOCH (2-amino-3-(3,4-dioxocyclohexa-1,5-dien-1-yl) propanoic acid), originated from Phe and Tyr residues. DOCH, being an electron acceptor for 1,4- or 1,6-type addition (i.e. Michael addition), was found responsible for new cross-links resulting in covalent aggregation of insulin during MCO. Specifically, 1,4- or 1,6-type addition products were detected between DOCH at positions B16, B26, A14, and A19, and free amino groups of the N-terminal amino acids Phe B1 and Gly A1, and side chains of Lys B29, His B5 and His B10. Fragments originating from peptide bond hydrolysis were also measured [10].

Chapter 5 describes studies on the anti-oxidant properties of several excipients during Cu^{2+}/ascorbate catalyzed oxidation of human insulin. Among the excipients studied, 100 µM triethylenetetramine (TETA) was the only one to inhibit almost completely oxidation-induced insulin aggregation, fragmentation and structural changes. TETA also prevented aggregation and fragmentation of a monoclonal IgG1 under identical oxidative conditions, indicating its general applicability as anti-oxidant for copper-sensitive proteins. In conclusion, TETA is a promising candidate excipient for formulations of oxidation-sensitive proteins.

Oxidation via Cu^{2+}/ascorbate of recombinant human interferon beta-1a (IFNβ) leads to highly immunogenic aggregates. In **Chapter 6** a study is presented on the identification of the oxidation sites and covalent cross-links in IFNβ exposed to MCO. Oxidation products of Met, His, Phe, Trp and Tyr residues were identified throughout the primary sequence. Similar to insulin (**Chapter 4**), covalent cross-links via 1,4- or 1,6-type addition between primary amines and DOCH were detected, while there was no evidence of disulfide bridge, Schiff base, or dityrosine formation. The chemical cross-links identified are most likely responsible for the formation of immunogenic covalent aggregates of IFNβ induced by oxidation.

In **Chapter 7** the immune mechanism responsible for the immunogenicity of aggregated recombinant human interferon alpha-2a (IFNα-2a) and the influence of aggregated IFNβ on IFNα's immunogenicity is studied. Transgenic mice immune tolerant for IFNα were treated with native or aggregated IFNα. After a washout period, the mice were rechallenged with aggregated or native IFNα to test for CD4+ T-cell involvement in immunogenicity. Furthermore, the mice were treated with a formulation containing aggregated IFNβ and native IFNα to test whether aggregated IFNβ acts as an adjuvant for IFNα. After a washout period and a rechallenge with either aggregated or native IFNα an apparent lack of immunological memory was observed. When we blocked CD4+ T-cell function in immune tolerant mice, aggregated rhIFNα failed to induce ADAs. Finally, immune tolerant mice treated with a mixture of aggregated IFNβ and native IFNα did not develop an antibody response against IFNα. These results are in line with previous results obtained with IFNβ [11].

Chapter 8 reports the validation of an adjuvant-free mouse model, which is intended as a tool to evaluate the immunogenicity of insulin formulations. Administration of human insulin (20 μg/dose, 12 doses over a period of 4 weeks) resulted in an immune response in non-transgenic animals, whereas the transgenic mice proved to be immune tolerant. This tolerance in transgenic mice could be circumvented when they were treated with insulin that was covalently bound to 50 nm polystyrene nanoparticles. This transgenic mouse model for human insulin was applied for comparing the immunogenicity of insulin formulations and may be of use to further investigate the mechanism behind the immunogenicity of therapeutic proteins, using insulin as a model protein.

Perspectives

The scientific work presented in this thesis aimed to contribute to the development of safer, less immunogenic biopharmaceuticals, by describing the mechanisms behind MCO-induced aggregation of therapeutic proteins and its relationship with immunogenicity. Further research is however needed to better prevent degradation of therapeutic proteins and better understand the factors contributing to their immunogenicity. This section provides some

perspectives for future research, focusing on: strategies to prevent protein degradation and analytical tools for protein characterization.

Strategies to prevent protein degradation

During the last two decades, industry became increasingly aware of the risk of degraded products, particularly aggregates, in protein formulations, justifying the search for solutions to prevent or reduce aggregation [3].

In general, two approaches can be employed to prevent protein degradation. The first is to improve protein stability by modifying the protein molecule, either by genetic engineering [12] or by chemical modification [13] (i.e. bio-conjugation like PEGylation). The second option is to improve stability by proper formulation [14]. An example of the first approach is the production of a human interleukin-1 where Asn36 is mutated into Ser36 [15]. The three-dimensional structure of this genetically engineered protein was shown to be identical to that of the native protein with dichroic studies and proton NMR. The mutant was resistant against base-catalyzed and temperature-induced deamidation [15].

Chemical modification, like conjugation of proteins with polymers (i.e. PEG), in some cases can be a solution as demonstrated for instance by the higher stability of PEGylated IFN proteins relative to their unmodified counterparts [16]. PEGylation has been used to increase the half life, resistance to enzymatic degradation and to prevent formation of fibrillar aggregates [17-19]. However, as shown in chapter 3, PEGylation did not prevent degradation of insulin in a liquid formulation. In our studies however we used a 5 kDa PEG, therefore we cannot exclude that other forms of PEGylation may improve the stability.

Excipients are widely employed to protect proteins from aggregation and oxidation [14]. In chapter 5, several anti-oxidant molecules were screened for their potential protective effect towards MCO-induced aggregation and fragmentation of insulin and a monoclonal antibody. Among the molecules screened triethylenetetramine [20] (TETA) turned out to be the most promising one in preventing MCO. However, it is currently unknown if TETA can prevent oxidation of amino acid residues not involved in aggregation, such as methionine and tryptophan, which are not present in insulin. Future studies, with the aid of tandem mass spectrometry, should verify the protective effect of TETA against oxidation of such amino acid residues.

Surfactant excipients, like polysorbate 80, are widely employed in protein formulation and useful for preventing aggregation [21]. One of the major drawbacks of polysorbates is that they contain alkyl chains that could auto-oxidize, generating protein-damaging peroxides. Given that peroxide formation is enhanced in aerobic environment and further promoted by light [21], a first strategy to reduce the amount of these reactive molecules in polysorbates would be preventing any contact with air/oxygen during storage.

Next, the addition of anti-oxidant excipients can be beneficial for inhibiting or minimizing generation of radicals. In this case chelating agents like TETA should not be used since these anti-oxidants are not able to scavenge radicals. Instead, molecules like methionine, vitamin E or glutathione could be considered, as shown by Ha et al [22]. Concluding, to prevent degradation a thorough understanding of the mechanisms involved is required. In chapter 4, for instance, by using high sensitive tandem mass spectrometry analysis and selective fluorogenic tagging [23-24], we showed that insulin aggregation under our oxidative system was due to tyrosine oxidation products that can react with amino groups in the insulin molecule. Based on these findings we further proposed two strategies to inhibit or reduce aggregation (chapter 4).

Similarly, mechanisms involved in several other pathways of degradation should be analyzed with the final goal of preventing any type of protein degradation.

Analytical tools for protein characterization

When a protein degrades, generally a heterogeneous mixture of degradation products with different physico-chemical features are formed, such as aggregates with different size, shape, protein conformation and chemical modifications, as well as structurally modified monomers and fragments [9]. Potentially any of these species can contribute to unwanted side reactions.

The availability of robust and reliable analytical tools capable of characterizing all of these species is required in research as well as for quality control (QC) purposes. For aggregate characterization, most of the routine analytical techniques approved for QC analysis, focus on the size range >1 µm and <100 nm, which obviously leave a big gap in the subvisible size range [25]. Techniques like nanoparticle tracking analysis [26] (NTA, used in several of the studies presented in this thesis) and flow imaging, allow visualization and

quantification of particulate aggregates and provide size information. These methods are increasingly used a characterization tools to fill the size gap previously mentioned [27]. Furthermore, an accurate mapping of chemical modifications of protein drugs can be achieved with high sensitive MS/MS analysis. Nonetheless, some chemical modifications like Trp oxidation can be artifact generated during sample preparation [28]. To prevent misinterpretation of the results, selective chemical derivatization (like the fluorogenic derivatization shown in chapter 4 that can be performed on oxidized Trp as well) before MS analysis, can be considered.

References

[1]   B. Leader, Q. J. Baca, D. E. Golan. Protein therapeutics: a summary and pharmacological classification. *Nat. Rev. Drug. Discov.* **2008**, *7*, 21-39.

[2]   Y. Vugmeyster, X. Xu, F. P. Theil, L. A. Khawli, M. W. Leach. Pharmacokinetics and toxicology of therapeutic proteins: Advances and challenges. *World J. Biol. Chem.* **2012**, *3*, 73-92.

[3]   W. Wang, S. K. Singh, N. Li, M. R. Toler, K. R. King, S. Nema. Immunogenicity of protein aggregates-concerns and realities. *Int. J. Pharm.* **2012**, *431*, 1-11.

[4]   V. Filipe, W. Jiskoot, A. H. Basmeleh, A. Halim, H. Schellekens. Immunogenicity of different stressed IgG monoclonal antibody formulations in immune tolerant transgenic mice. *MAbs* **2012**, *4,* 740-752.

[5]   S. Hermeling, L. Aranha, J. M. A. Damen, M. Slijper, H. Schellekens, D. J. A. Crommelin, W. Jiskoot. Structural characterization and immunogenicity in wild-type and immune tolerant mice of degraded recombinant human interferon alpha2b. *Pharm. Res.* **2005**, *22*, 1997-2006.

[6]   S. Hermeling, H. Schellekens, C. Maas, M. F. Gebbink, D. J. Crommelin, W. Jiskoot. Antibody response to aggregated human interferon alpha2b in wild-type and transgenic immune tolerant mice depends on type and level of aggregation. *J. Pharm. Sci.* **2006**, *95*, 1084-1096.

[7]   M. M. van Beers, M. Sauerborn, F. Gilli, V. Brinks, H. Schellekens, W. Jiskoot. Oxidized and aggregated recombinant human interferon beta is immunogenic in human interferon beta transgenic mice. *Pharm. Res.* **2012**, *28*, 2393-2402.

[8]   E. R. Stadtman. Metal ion-catalyzed oxidation of proteins: biochemical mechanism and biological consequences. *Free. Radic. Biol. Med.* **1990**, *9*, 315-325.

[9]   R. Torosantucci, B. Kukrer, A. Mero, M. Van Winsen, R. Tantipolphan, W. Jiskoot. Plain and mono-pegylated recombinant human insulin exhibit similar stress-induced aggregation profiles. *J. Pharm. Sci.* **2011**, *100*, 2574-2585.

[10]   R. Torosantucci, O. Mozziconacci, V. Sharov, C. Schöneich, W. Jiskoot. Chemical Modifications in Aggregates of Recombinant Human Insulin Induced by Metal-Catalyzed Oxidation: Covalent Cross-Linking via Michael Addition to Tyrosine Oxidation Products. *Pharm. Res.* **2012,** 29, 2276-2293

[11]   M. M. van Beers, M. Sauerborn, F. Gilli, V. Brinks, H. Schellekens, W. Jiskoot. Aggregated recombinant human interferon Beta induces antibodies but no memory in immune-tolerant transgenic mice. *Pharm. Res.* **2010**, *27*, 1812-1824.

[12]   M. Kamionka. Engineering of therapeutic proteins production in Escherichia coli. *Curr. Pharm. Biotechnol .***2011**, *12*, 268-274.

[13]   S. Jevsevar, M. Kunstelj, V. G. Porekar. PEGylation of therapeutic proteins. *Biotechnol. J.* **2010**, *5*, 113-128.

[14]   W. Wang. Instability, stabilization, and formulation of liquid protein pharmaceuticals. *Int. J. Pharm.* **1999**, *185*, 129-188.

[15]   P. T. Wingfield, R. J. Mattaliano, H. R. MacDonald, S. Craig, G. M. Clore, A. M. Gronenborn, U. Schmeissner. Recombinant-derived interleukin-1 alpha stabilized against specific deamidation. *Protein. Eng.* **1987**, *1*, 413-417.

[16]   A. Basu, K. Yang, M. Wang, S. Liu, R. Chintala, T. Palm, H. Zhao, P. Peng, D. Wu, Z. Zhang, J. Hua, M. C. Hsieh, J. Zhou, G. Petti, X. Li, A. Janjua, M. Mendez, J. Liu, C. Longley, M. Mehlig, V. Borowski, M. Viswanathan, D. Filpula. Structure-function engineering of interferon-beta-1b for improving stability, solubility, potency, immunogenicity, and pharmacokinetic properties by site-selective mono-PEGylation. *Bioconjug. Chem.* **2006**, *17*, 618-630.

[17]   K. Hinds, J. J. Koh, L. Joss, F. Liu, M. Baudys, S. W. Kim. Synthesis and characterization of poly(ethylene glycol)-insulin conjugates. *Bioconjug. Chem.* **2000**, *11*, 195-201.

[18]   K. D. Hinds, S. W. Kim. Effects of PEG conjugation on insulin properties. *Adv. Drug. Deliv. Rev.* **2002**, *54*, 505-530.

[19]   T. Uchio, M. Baudys, F. Liu, S. C. Song, S. W. Kim. Site-specific insulin conjugates with enhanced stability and extended action profile. *Adv. Drug. Deliv. Rev.* **1999**, *35*, 289-306.

[20]   G. J. Cooper. Therapeutic potential of copper chelation with triethylenetetramine in managing diabetes mellitus and Alzheimer's disease. *Drugs* **2011**, *71*, 1281-1320.

[21]   B. A. Kerwin. Polysorbates 20 and 80 used in the formulation of protein biotherapeutics: Structure and degradation pathways. *J. Pharm. Sci.* **2008**, *97*, 2924-2935.

[22]   E. Ha, W. Wang, Y. J. Wang. Peroxide formation in polysorbate 80 and protein stability. *J. Pharm. Sci.* **2002**, *91*, 2252-2264.

[23]   V. S. Sharov, E. S. Dremina, N. A. Galeva, G. S. Gerstenecker, X. Li, R. T. Dobrowsky, J. F. Stobaugh, C. Schöneich. Fluorogenic Tagging of Peptide and Protein 3-Nitrotyrosine with 4-(Aminomethyl)-benzenesulfonic Acid for Quantitative Analysis of Protein Tyrosine Nitration. *Chromatographia* **2010**, *71*, 37-53.

[24]   V. S. Sharov, E. S. Dremina, J. Pennington, J. Killmer, C. Asmus, M. Thorson, S. J. Hong, X. Li, J. F. Stobaugh, C. Schöneich. Selective fluorogenic derivatization of 3-nitrotyrosine and

3,4-dihydroxyphenylalanine in peptides: a method designed for quantitative proteomic analysis. *Methods Enzymol.* **2008**, *441*, 19-32.

[25]   J. F. Carpenter, T. W. Randolph, W. Jiskoot, D. J. Crommelin, C. R. Middaugh, G. Winter, Y. X. Fan, S. Kirshner, D. Verthelyi, S. Kozlowski, K. A. Clouse, P. G. Swann, A. Rosenberg, B. Cherney. Overlooking subvisible particles in therapeutic protein products: gaps that may compromise product quality. *J. Pharm. Sci.* **2009**, *98*, 1201-1205.

[26]   V. Filipe, A. Hawe, W. Jiskoot. Critical evaluation of Nanoparticle Tracking Analysis (NTA) by NanoSight for the measurement of nanoparticles and protein aggregates. *Pharm. Res.* **2010**, *27*, 796-810.

[27]   J. den Engelsman, P. Garidel, R. Smulders, H. Koll, B. Smith, S. Bassarab, A. Seidl, O. Hainzl, W. Jiskoot. Strategies for the assessment of protein aggregates in pharmaceutical biotech product development. *Pharm. Res.* **2011**, *28*, 920-933.

[28]   I. Perdivara, L. J. Deterding, M. Przybylski, K. B. Tomer. Mass spectrometric identification of oxidative modifications of tryptophan residues in proteins: chemical artifact or post-translational modification? *J. Am. Soc. Mass. Spectrom.* **2010**, *21*, 1114-1117.