Oxidation, aggregation and immunogenicity of therapeutic proteins

Torosantucci, R.

Chapter 2

Oxidation of therapeutic proteins and peptides: structural and biological consequences

Riccardo Torosantucci[1], Christian Schöneich[2], Wim Jiskoot[1]

[1]Division of Drug Delivery Technology, Leiden Academic Centre for Drug Research (LACDR), Leiden University, Leiden, the Netherlands.

[2]Department of Pharmaceutical Chemistry of Kansas University, Lawrence, Kansas, USA.

Submitted for publication

Abstract

Oxidation is a common degradation pathway that affects therapeutic proteins and peptides during production, purification, formulation, transportation, storage and handling of solid and liquid preparations. In the present work we review the scientific literature about structural and biological consequences of protein/peptide oxidation. Representative examples are discussed of specific products whose oxidation has been recently studied, including monoclonal antibodies, calcitonin, granulocyte colony-stimulating factor, growth hormone, insulin, interferon alpha and beta, oxytocin and parathyroid hormone. These examples illustrate that oxidation often leads to modifications of higher-order structures, including aggregate induction, and can generate products that are pharmacokinetically different, biologically less active and/or potentially more immunogenic than their native counterpart. It is therefore crucially important during the pharmaceutical development of therapeutic proteins and peptides to comprehensively characterize oxidation products and evaluate the impact of oxidation-induced structural modifications on the biological properties of the drug.

Introduction

In the last thirty years proteins and peptides have gained importance in the treatment of a broad number of diseases for which no other therapy is available [1]. Instability, however, represents a serious problem in the development of therapeutic proteins and peptides [2].

In particular oxidation, which has been reported to occur during production [3], purification [4], formulation [5] and storage [6], is a major concern [7], as it can extensively modify the primary structure of proteins and peptides, by which changes in secondary, tertiary and quaternary structure may arise [8-10]. Whereas there are several excellent reviews describing oxidation mechanisms [11], products of amino acid oxidation [12-16], the biochemical basis of protein oxidation [17-18] and strategies to prevent oxidation [2, 11-12, 17], to the best of our knowledge, only one review, published twenty years ago, described the pharmaceutical consequences of protein oxidation [19]. At that time, however, experimental data about biological consequences of oxidation were scarce.

Here we aim to give an update on the current knowledge about the consequences of oxidative modification for amino acid residues (i.e. primary structure), higher-order structures (i.e. secondary, tertiary and quaternary structure), biological activity, half-life and immunogenicity of several protein and peptide therapeutics.

After briefly introducing the potential causes of oxidation during production, purification, formulation and storage, we will discuss the consequences of oxidation for: monoclonal antibodies (mAbs), calcitonin (CT), granulocyte colony-stimulating factor (G-CSF), growth hormone (GH), insulin, recombinant human interferon alpha-2a (IFNα-2a) interferon alpha-2b (IFNα-2b) and interferon beta-1a (IFNβ-1a), oxytocin and parathyroid hormone (PTH).

Oxidation of proteins and peptides

Most biopharmaceuticals are produced by recombinant DNA technologies, usually by employing microbial hosts like E. coli [20] or mammalian cells like Chinese hamster ovary (CHO) cells [21]. Already during the production steps, the concentration of dissolved oxygen (DO) can influence the oxidative state of therapeutic proteins, as demonstrated for the production in E. coli of recombinant human IFNγ, where an increase in carbonyl groups [18] (a general

marker of oxidative modification) correlated with a relatively high DO concentration (i.e. 60% DO), suggesting that the aerobic environment should be scrupulously monitored [3]. However, also low oxygen concentration (a condition known as hypoxia) may induce oxidative stress through the production of reactive oxygen species in mammalian host cells, likely generated by electrons leaking from the mitochondrial electron transport chain [22-25]. In support of this, oxidation-induced fragmentation of recombinant human IgG1 produced in CHO cells was observed in the purified material and, interestingly, the same degradation was reproduced by *in vitro* incubation of the protein with hydrogen peroxide [26].

Besides oxidation that may arise during the production in cell culture, oxidation can occur in the subsequent downstream processes. For instance, purification of lactate dehydrogenase, using metal affinity chromatography, yielded an oxidized product [4].

During formulation and storage several excipients and impurities can directly or indirectly favor oxidation. For instance, hydrogen peroxide has been encountered as an impurity in polymeric excipients such as polyethylene glycol (PEG) or polysorbate [5, 27]. Additionally, these polymeric excipients can spontaneously oxidize in aerobic environment, without the aid of a catalyst (auto-oxidation) [28-29], generating several peroxides. Among the impurities which might favor oxidation, transition metals represent a common threat, as they can catalyze oxidation reactions [14] already at submicromolar concentration [30]. Furthermore, transition metals being air pollutants [31-32] may contaminate buffers [33] and excipients such as sugars, surfactants and amino acids [34]. Also, they can be released from containers [11], making it difficult to fully avoid their presence in formulations.

Chemical modifications in amino acids induced by oxidation

Potentially all 20 natural amino acids can be oxidized [16], however, cysteine (Cys), histidine (His), methionine (Met), phenylalanine (Phe), tryptophan (Trp) and tyrosine (Tyr) are generally most prone to oxidation, due to the high reactivity of sulfur atoms and aromatic rings towards various reactive oxygen species [12]. Table I provides a comprehensive summary of reported cases of protein/peptide oxidation, including the chemical changes in primary and higher-order structures, as well as observed biological consequences.

Table I. Summary of reported structural and biological consequences of oxidation of protein and peptide therapeutics.

Protein [a]	Oxidative stress [b]	Chemical changes in primary structure [c]	Changes in higher-order structure	Biological consequences	Ref.
IgG1 (humanized monoclonal)	H_2O_2	M252 (HC [d]) M428 (HC) M358 (HC)	Reduced melting temperature (Tm) of CH2 domain	Decreased binding to FcRn	49
	H_2O_2	M252 (HC) M428 (HC)	Subtle conformational changes	Reduction of serum half-life	50, 52
IgG1 Fc (human, E. coli derived)	H_2O_2	M33 (HC) M209 (HC)	Altered secondary and tertiary structure Reduced melting temperature of CH2 domain	n.i. n.i.	51
IgG1 (not specified)	H_2O_2	M34 (HC) M83 (HC) M253 (HC) M429 (HC) M4 (LC)	Large structural change in the region of heavy chain residues 247–253 Smaller changes in CH2 and CH3 domains	n.d.	54, 55
IgG1 (human monoclonal)	TBHP	M252 (HC) M428 (HC)	Changes in secondary and tertiary structure Formation of (mainly submicron) aggregates	Decreased binding affinity for protein A, protein G and FcRn	48, 38

IgG1 (HER2)	TBHP, H_2O_2 and storage	M83 (HC) M253 (HC) W32 (LC)	Aggregation and fragmentation	n.i.	59
IgG1 (humanized MEDI-493)	ultraviolet (UV) light irradiation	W105 (HC)	No significant changes in secondary structure	Loss of target binding and biological activity	60
IgG1 (fully human)	production in CHO cell culture, H_2O_2	M C H	Hinge region fragmentation	n.i.	26
IgG1	Cu^{2+}/ascorbate	n.i.	Changes in secondary and tertiary structure Aggregation	Immunogenic in transgenic immune tolerant mice	56
IgG2	Cu^{2+}/ascorbate	M34 (HC) M82 (HC) M246 (HC) M352 (HC) M391 (HC) M422 (HC) H304 (HC) H427 (HC) W156 (HC)	Changes in secondary and tertiary structure Aggregation	n.i.	57, 58
	H_2O_2	M34 (HC), M246 (HC), M352 (HC), M391 (HC)	Changes in secondary and tertiary structure Aggregation	n.i.	57, 58

	Thermal stress	M34 (HC) M246 (HC) M422 (HC)	Changes in secondary and tertiary structure Aggregation	n.i.	57, 58
	Mechanical stress	M34 (HC) M82 (HC) M246 (HC) M352 (HC) M391 (HC) M422 (HC) W36 (LC) W47 (HC) W98 (LC) W156 (HC) W271 (HC)	Changes in secondary and tertiary structure Aggregation	n.i.	57, 58
sCT	H_2O_2	n.i.	Formation of fibrillar aggregates	n.i.	66
	Storage	C	Dimerization involving Cys residues and trisulfide variants	n.d.	67
	Fe^{2+}/ascorbate/light	n.i.	Changes in secondary structure Formation of fibrillar aggregates	n.i.	61
G-CSF	H_2O_2	M1, M122, M127, M138	Conformational changes	Altered biological activity and serum half life	68, 69

	TBHP	M1	Conformational changes	Altered biological activity and serum half life	69
		M122			
		M127			
		M138			
GH	H_2O_2	M14	Subtle conformational changes	Minor effect on receptor binding and *in vitro* biological potency	73
		M125			
	H_2O_2	M14	Subtle conformational changes	Decreased thermal stability	74
		M125			
	Cu^{2+}/ascorbate	H18	Subtle conformational changes Formation of dimers	n.i.	80
		H21			
	AAPH	M14	Aggregation	n.i.	78
		M125			
		M170			
		L101			
		Y103			
Insulin	Storage	C	Aggregation	n.i.	83
	Cu^{2+}/ascorbate	H (B5, B10)	Changes in secondary and tertiary structure Aggregation	Immunogenic in transgenic immune tolerant mice	9, 10
		F (B1, B24, B25)			
		Y (A14, A19, B16, B26)			

	Cu^{2+}/H_2O_2 And in vitro incubation with plasma from diabetic patients	F and Y oxidation products	n.i.	Decreased hypoglycemic activity	96
	Cu^{2+}/H_2O_2	F and Y oxidation products	Aggregation	Decrease in the insulin-dependent glucose utilization by human adipose tissue	97
IFN alpha-2a	H_2O_2	n.i.	n.i.	Reduced biological activity	99
	Storage	n.i.	n.i.	Enhanced immunogenicity	100, 101
	Cu^{2+}/ascorbate	n.i.	Changes in secondary structure Aggregation	Immunogenic in transgenic immune tolerant mice	(e)
IFN alpha-2b	Cu^{2+}/ascorbate	M16 M21 M148	Changes in secondary structure Aggregation	Immunogenic in transgenic immune tolerant mice	105
	Storage	M111	Changes in secondary structure	n.d.	104

IFN beta-1a	Storage	C	Disulfide linked aggregates	n.i.	107
	Cu^{2+}/ascorbate	n.i.	Structural changes Aggregation	n.i.	109
Oxytocin	Storage at different pH and temperature	C Y	Tri- and tetra sulfide derivatives Dityrosine linked dimers	n.i.	112
PTH	Storage	n.i.	Changes in secondary structure Aggregation	n.i.	116
	H_2O_2	M8 M18	Changes in secondary structure	n.i.	118
	H_2O_2	M8 M18	n.i.	Reduced biological activity	119
bPTH	H_2O_2	M8	n.i.	Reduced biological activity	121
pPTH	H_2O_2	M8	n.i.	Reduced biological activity	122

a: sCT: salmon calcitonin; G-CSF: granulocyte colony-stimulating factor; GH: growth hormone; PTH: parathyroid hormone.
b: TBPH: tert-butylhydroperoxide; AAPH: 2,2′- azobis(2-methylpropionamidine).
c: Amino acid residues in one letter code: C: cysteine; F: phenylalanine; H: histidine; M: methionine; W: tryptophan; Y: tyrosine.
d: Abbreviations: A and B: insulin chain A and chain B, respectively; HC and LC: IgG heavy chain and light chain, respectively; n.d.: not detected; n.i.: not investigated.
e: unpublished data by Sauerborn et al.

As oxidative modifications at the amino acid level have been extensively reviewed elsewhere [12-13, 16], we focus the discussion below on the higher-order structural consequences and the biological consequences observed for several representative protein and peptide drugs.

Consequences of protein and peptide oxidation

Monoclonal antibodies

All human IgGs feature a characteristic "Y" shape [35]: the lower part contains a single crystallizable region (Fc) critical for effector functions and half-life [36]. The upper part consists of two identical regions (Fab) that contain the complementarity determining regions (CDRs) responsible for antigen binding [37]. Most mAbs belong to the IgG1 and IgG2 subclasses, which share 97% of sequence homology [38]. The Fc region can contain up to four Met residues: Met residues at positions 252 and 428 (based on the Eu numbering system [39]) are conserved in all IgGs [40], the presence of Met 358 in IgG1 is dependent on the allele of the gene [41], while Met 397 is only present in IgG2 and IgG3 [42]. Modification of any of these Met residues may adversely affect the Fc-dependent effector function of mAbs [42].

Several authors investigated the susceptibility to oxidation of Met residues under different stress or storage conditions [43-47]. However, few studies reported the consequences of such modifications on protein structure and pharmacokinetics.

Oxidation of Met 252 and Met 428 reduced the binding with Protein A [48] (a protein often used in affinity chromatography) and the neonatal Fc receptor (FcRn) [38, 49]. This can reduce the biological half-life of the antibody, as shown by Wang et al. [50], who demonstrated that a mAb containing 80% of oxidized Met 252 features more than 4-fold reduction in the half-life in transgenic mice with human FcRn. When the percentage of Met oxidation was lower, i.e. 40%, the measured half-life was comparable to that of the native mAb.

Liu et al. [51] noticed that hydrogen peroxide-induced oxidation of Met residues in E. coli-expressed Fc, resulted in alteration of secondary and tertiary structure, evaluated by circular dichroism spectroscopy, and in a reduced melting temperature of the CH2 domain (note that Liu et al. referred to Met 33 and Met 209, which correspond to Met 252 and Met 428 on the

intact heavy chain sequence). It must be mentioned that the Fc used was produced in E. coli and thus lacks glycosylation, which is important for protein stability: Met oxidation in a glycosylated IgG1 led to similar changes in the thermal stability but conformational changes of the antibody with oligosaccharides where minor, indicating a partial protective effect of the sugar moiety [52-53]. Interestingly, also the deamidation rate of Asn 67 and Asn 96 increased, likely as a result of Met oxidation-mediated conformational changes [51]. Destabilization of the α-helix of the residues 247-253 of the Fc region of IgG1 was also observed upon hydrogen peroxide treatment [54-55]. These findings suggest that oxidation of Met residues can result in conformational changes of mAbs.

Metal catalyzed oxidation (MCO) of a monoclonal human IgG, induced by Cu^{2+}/ascorbate, generated mainly micron-sized aggregates with secondary and tertiary structure alterations that were immunogenic in a transgenic, immune-tolerant mouse model [56]. A monoclonal IgG2 was evaluated under similar stress conditions and the authors observed severe changes in secondary and tertiary structure, associated with the site specific oxidation of His 304 and His 427, besides oxidation of several Met residues and of Trp 156 [57-58]. Also particles in the size range between 0.2-10 μm were detected.

Similarly, oxidation of IgG2 with hydrogen peroxide modified the higher order structural properties of the protein and induced the formation of polydisperse aggregates. In addition to Met oxidation, Trp oxidation was observed but not the oxidation of His [57-58].

Hensel et al. suggested that the oxidation of Trp 32 (in the CDR region of the light chain) was mainly responsible for the progressive loss of target binding and biological activity [59]. Similarly, the oxidation of Trp 105, a residue in the CDR3 of the heavy chain of a humanized mAb against respiratory syncytial virus, was considered responsible for the activity loss [60]. Altogether, these results demonstrate that Met is only one of the potential targets of oxidation and oxidation frequently compromises the conformation and biological functions of monoclonal IgGs.

Calcitonin

CT is a polypeptide hormone of 32 amino acids which, in aqueous solution, assumes an unstructured conformation [61]. Mainly human and salmon

calcitonin (hCT and sCT, respectively) are used for therapeutic purposes. The two polypeptides share only 50% sequence homology, nonetheless higher order structural features are similar between the two hormones [61]. In aqueous solution, hCT tends to aggregate faster than sCT, causing the formation of fibrillar precipitates [61].

Aggregated and oxidized forms of hCT were observed *in vivo* in plasma under non-pathological conditions [62], justifying studies on the consequences of CT oxidation. Although CT contains Met, His, Phe and Tyr residues, all of which are potential oxidation targets, oxidation (during storage or forced oxidative stress) of this polypeptide hormone appears to affect mainly Met 8, the only Met residue available. Reduction of bioactivity was observed upon oxidation of Met 8 [63-64]; however, more recently it was found that the aggregation rate of Met oxidized hCT decreased [65], illustrating that oxidation not necessarily accelerates aggregation.

Aggregation of sCT accompanied by alteration of secondary structure was observed upon hydrogen peroxide treatment [66]: this suggests that mild oxidative conditions are capable of inducing structural changes in sCT.

Dimers involving Cys residues as well as a trisulfide derivative were measured in a different study investigating the stability of the hormone in aqueous solutions [67]. These findings suggest that aggregation involving disulfide scrambling of the thiol groups can be involved in sCT aggregation.

When testing the effect of hydroxyl radicals generated via a modified Fenton reaction (60-W tungsten lamp in combination with ferrous sulfate and ascorbic acid), sCT amyloid aggregates were detected. Interestingly, they were structurally similar to what was observed *in vivo* for hCT, in carcinoma medullary plaques [61].

In conclusion, *in vitro* oxidation of CT might produce fibrillar aggregates similar in structure as those observed *in vivo*. Met and Cys residues seem to be responsible for the observed structural changes. However, it is still poorly investigated if oxidation of His and Tyr, both present in hCT as well as sCT, can occur and contributes to aggregation or structural changes of this polypeptide.

Granulocyte colony-stimulating factor

Recombinant human G-CSF contains 175 amino acid residues, several of which are susceptible to oxidation [68].

Simultaneous oxidation of all four Met residues (in position 1, 122, 127 and 138) resulted in a dramatic decrease of the biological activity to 3% [69]. The biological activity of the HPLC fraction containing G-CSF with only Met 1 oxidized, was largely retained (i.e. 80% relative to G-CSF prior to oxidation), indicating that this residue is less important for the activity. In addition, engineered variants of G-CSF, where either Met 127 or Met 138 was replaced by leucine (Leu), were still sensitive to oxidation-induced inactivation. However, the variant with Leu replacement at both sites was more stable and retained *in vitro* biological activity following oxidative stress. All these experiments suggest that oxidation of Met 127 and Met 138 accounted for most of the activity loss [69].

Besides oxidation of Met residues in G-CSF, Cys oxidation is a point of concern. Under physiological conditions (37 °C, pH 7.0), G-CSF showed a significant propensity to aggregate. Several studies demonstrated that the free Cys in position 17, upon oxidation, forms a new disulfide bridge that is responsible for G-CSF aggregation [68, 70-71].

Growth hormone

Oxidative modifications of recombinant human growth hormone (hGH) have been widely described (Table I), mainly with respect to Met oxidation.

Relatively mild oxidative conditions, attained during exposure to hydrogen peroxide, have been reported to lead to selective generation of Met sulfoxides from the two most accessible Met residues in hGH (Met 14 and Met 125). Although this does not seem to induce gross conformational changes [72-73], the thermal stability of the protein dropped [74]. This may be due to the generation of Met 14 and Met 125 sulfoxides, which increases the polarity and the size of these amino acids; furthermore the new hydrogen bond networks that the protein can establish, may contribute to the observed decrease in thermal stability [74].

Cunningham et al. showed that Met 14 contributes only slightly to the binding of the hormone to its receptor [75]. In agreement with this study, the oxidation of Met 14 and Met 125 was reported to have little effect on hGH's receptor affinity and potency [73].

In contrast, Met 170 is located within the core of the native protein [76]. Nevertheless, the mass spectrometric analysis of a marketed hGH product

(Genotropin®, expressed in *E. coli K12*) revealed that 2% of the expressed protein contains several chemical modifications, including Met 170 sulfoxide [77]. This residue is located on the alpha helix IV of hGH, which is involved in one of the two receptor binding sites [76].

Steinmann et al. detected the oxidation of Met 170 (together with that of Met 14 and Met 125) during the exposure of hGH to peroxyl radicals generated from 2,2′-azobis(2-methylpropionamidine) (AAPH) [78]. In addition, the authors detected di-tyrosine, Leu 101 hydroperoxide and several oxidation products of Tyr 103. These oxidation conditions led to the formation of dimers (21%) and trimers (13%).

Light exposure resulted in the selective oxidation of His 21 [79]. Furthermore, MCO, induced by exposure to Cu^{2+}/ascorbate, specifically modified His 18 and His 21, which are both located on helix I and are critical for the integrity of the metal binding site of this hormone [80-81].

Insulin

One of the first reported experiments involving insulin oxidation dates back to 1948 when Frederick Sanger employed a mixture of hydrogen peroxide and formic acid, which generates performic acid, to fractionate insulin's A and B chains. Previously reduced Cys residues were oxidized to cysteic acid and also Tyr oxidation products were observed [82]. Since then, insulin oxidation has been extensively investigated.

Covalent aggregation of lyophilized insulin was observed upon storage at different temperatures and moisture contents [83]. Reduction of the native disulfide bridge followed by re-oxidation was responsible for new intermolecular disulfide bridges that mediate aggregate formation. Furthermore, aggregation involving Cys residues does not necessarily require the presence of this amino acid in its reduced form (i.e. free thiol groups) [83].

Therapeutic formulations of insulin, in solution or in suspension, analyzed after long term stability studies contained dimers and oligomers resulting from reduction-oxidation of Cys residues [83]. Formation of insulin aggregates with altered 3D structure was observed upon MCO using Cu^{2+}/ascorbate. In particular, the Tyr oxidation products 3,4-dihydroxyphenylalanine (DOPA) and 2-amino-3-(3,4-dioxocyclohexa-1,5-dien-1-yl) propanoic acid (DOCH) were observed [9]. The latter, being an electrophile, was shown to be involved

in covalent cross-links with several amino groups of the insulin molecule, which led to new intra- and intermolecular cross-links. This oxidized and aggregated insulin induced anti-insulin antibodies when injected in transgenic mice immune tolerant for human insulin (unpublished data). MCO also led to the oxidation of His B5 and B10, which are important binding sites for zinc ions that play a central role in the formation of insulin's quaternary structure [84-85].

In the presence of zinc ions, insulin exists as hexamers [86], which are the main components in several long-acting therapeutic insulin formulations [87]. Oxidative stress that targets insulin's His residues involved in zinc ion binding can therefore result in unexpected pharmacokinetics.

Several studies have investigated the oxidative modifications that insulin can undergo in diabetic patients. Subjects affected by diabetes have generally high glucose plasma concentrations (hyperglycemia) and display oxidative stress associated with a decrease in the concentration of biological antioxidants such as reduced glutathione (GSH) [88]. The former event is responsible for glycation of insulin, i.e. the formaton of a covalent adduct between insulin and glucose, where insulin is oxidized (loss of hydrogen atoms from amino groups).

Glycated insulin has been measured *in vivo* and its biological activity was decreased [89]. Another important consequence of hyperglycemia is the generation of α-oxoaldehydes like glyoxal, methylglyoxal and 3-deoxyglucosone [90], which can react with insulin generating aggregates of oxidized insulin [91].

Furthermore oxidative stress is responsible for lipid peroxidation of n-3 and n-6 polyunsaturated fatty acids, which eventually generates reactive aldehydes such as 4-hydroxy-2-hexenal and 4-hydroxy-2-nonenal [92]. The reaction between insulin and these reactive aldehydes (α, β unsaturated carbonyl compounds) occurs through Michael addition and introduces new carbonyl groups in the insulin molecule [92]. Glucose uptake as well as the hypoglycemic effect in mice was significantly reduced after treatment with insulin oxidized with reactive aldehydes, compared to treatment with native insulin [92].

More recently, based on the hypothesis that the plasma copper ion concentration is higher in diabetic patients than in normal subjects, Cheng et

al. studied the copper induced catalyzed oxidation of glycated insulin, which yielded aggregates, fragments and oxidation products [93].

Similarly, Guedes et al. investigated MCO of glycated insulin using the Fenton reaction, which induced aggregation and fragmentation of oxidized glycated insulin [94]. It is noteworthy that besides oxidation, insulin was found to be glycated on several sites including the N-terminal Gly A1, which is important for the biological activity [94-95].

Montes-Cortes et al. [96] discovered that the incubation of insulin with plasma from diabetic patients resulted in Tyr oxidation products, increased carbonyl content and decreased biological activity, similar to what observed upon Fenton oxidation of insulin [96-97]. These results suggest that a correlation between *in vivo* and *in vitro* oxidation may exist and that oxidative modifications on the insulin molecule can decrease the biological activity of this polypeptide hormone.

Interferon alpha

During storage, particularly at neutral and acidic conditions, IFNα-2a is known to undergo oxidation of Met residues [98].

Hydrogen peroxide-induced oxidation generated an IFNα-2a variant that featured reduced specific biological activity [99], but the sites of oxidation were not determined.

Immunogenicity of oxidized and aggregated IFNα-2a, formulated as lyophilized powder and stored at ambient temperature, was evaluated in patients: the oxidized form was more immunogenic than several other formulations of non-oxidized rhIFNα-2a [100-101]. Recently IFNα-2a oxidized by Cu^{2+}/ascorbate was found to undergo structural modifications and aggregation; this product was immunogenic in a transgenic mouse model immune-tolerant for human IFNα-2a (unpublished results).

In IFNα-2b, all of the 5 Met residues are sensitive to oxidation in solution under different tested storage conditions [102]. Here, Met 111 oxidizes very easily and IFNα-2b containing oxidized Met 111 has been detected in a cream for topical use [103]. The alpha-helical content of the protein containing oxidized Met 111 was slightly decreased parallel to an increase in the beta-sheet contribution; however, the biological activity was not affected [104].

MCO of IFNα-2b, where Met 16, Met 21 and Met 148 were converted into the sulfoxide derivatives, generated aggregates that were immunogenic in the transgenic immune tolerant mouse model mentioned above [105].

Interferon beta

Under mild oxidative conditions, achieved with hydrogen peroxide, Orru et al. [106] observed the oxidation of the surface exposed Met 117 in IFNβ-1a, which was the most reactive Met residue, followed by the oxidation of Met 36 and Met 1. Cys residues and the carbohydrate moiety were not modified and the biological activity of the protein was fully retained, pointing to minor consequences of Met oxidation for the activity of this cytokine [106].

Free Cys 17 in IFNβ-1a can be involved in redox chemistry as demonstrated by the detection of 2% of disulfide linked aggregates after prolonged storage [107].

Furthermore, deglycosylated IFNβ-1a was more sensitive to formation of insoluble, disulfide-linked aggregates with diminished biological activity, indicating a protective role of the carbohydrate moiety [108].

The oxidation of IFNβ-1a with Cu^{2+}/ascorbate generated covalent aggregates that contained native-like epitopes, had an average diameter of 1.6 μm and were immunogenic in transgenic mice immune tolerant for human IFNβ [109]. These aggregates were shown to be cross-linked through 1,4 and 1,6-type addition at Tyr oxidation products [110]. Oxidation mediated by hydrogen peroxide of IFNβ-1a also yielded immunogenic IFNβ-1a aggregates, but the percentage of monomeric IFNβ-1a was higher compared to the MCO protein [109].

Oxytocin

Oxytocin is a small peptide which contains a six-amino acid ring (Cys1, Tyr2, Ile3, Gln4, Asn5, Cys6) and a tail of three amino acids (Pro7, Leu8, Gly9-NH2) [111]. As for CT, oxytocin formed tri- and tetrasulfide derivatives (introduction of one and two sulfur atoms, respectively, into its chemical structure) under accelerated degradation conditions at different pH and temperature [112].

Besides degradation involving sulfur atoms, heat stressed oxytocin formulations at pH 4.5, 7.0 and 9.0, generated also di-tyrosine-linked dimers, albeit at low percentages [112].

Rosei et al. showed that Tyr 2 in the oxytocin molecule, even though it is located internally in the primary sequence, functions better as hydrogen donor than free Tyr [113]. Hence, Tyr radicals, which are precursors in the generation of di-tyrosines [114], can be easily formed in the oxytocin molecule.

Parathyroid hormone

Synthetic parathyroid hormone contains two Met, three His, one Trp and one Phe, residues which are particularly oxidation-sensitive under several applied experimental conditions [34].

Oxidation of this hormone has been detected in blood from patients with renal disease [115]. During long term storage of hPTH (1-34) up to 24 weeks at room temperature, oxidation-induced aggregation and loss of secondary structure were observed. Although the oxidation sites were not determined, it was found that sucrose substantially reduced hPTH (1-34) oxidation. This protective effect was due to a more compact conformation that the hormone assumed in presence of the sugar, where amino acid residues sensitive to oxidation are more buried [116].

hPTH (1-34) features minimal tertiary structure [117], but the secondary structure is well defined and consists of approximately 33% alpha-helical content and 32% ß-sheet [118]. Circular dichroism spectroscopic studies indicated that most of the secondary structure resides in the N-terminal region of this hormone, in agreement with the findings that oxidation of Met 8, close to the N-terminal region, produces substantial changes, while oxidation of Met 18 has a small impact on the secondary structure [118]. This finding correlated well with the observed alteration in biological activity: oxidation of Met 8, caused a remarkably larger suppression of the activity when compared to that of Met 18 [119].

More recently it was discovered that oxidation of Met 8 (into a sulfide radical cation during Fenton oxidation) results in the specific hydrolysis of the peptide bond between Met 8 and His 9, suggesting that also fragmentation of PTH (1-34) can occur during oxidation catalyzed by iron (II) [120].

A similar decreased activity by oxidation was observed for bovine [121] and porcine PTH, which both share Met 8 (but not Met 18) with the human counterpart [122]. Thus, the region around Met 8 is important for the activity. Nonetheless Met 18 in human PTH is another receptor recognition site [123], which would explain why the activity further decreased when both Met 8 and Met 18 are oxidized [119]. Based on these results the native secondary structure seems to be essential for receptor binding, as it is strongly perturbed upon oxidation of Met residues [118]. Additional studies indicated that Met 18 oxidizes more easily than Met 8, probably because PTH assumes a secondary structure that protects Met 8 against oxidation. Indeed, unfolding of the protein with 3 M guanidinium hydrochloride eliminated this difference, as it generates similarly surface-exposed Met residues [124].

Conclusion

The structural and biological consequences of several therapeutic proteins and peptides were reviewed. Redox chemistry of Cys residues is widely involved in the generation of new intra- and intermolecular covalent bonds, as observed for IFNβ-1a, insulin, calcitonin and oxytocin.

Met oxidation usually involves solvent-exposed residues and often results in altered protein conformation and biological activity, even when the aggregation state of the protein is not affected.

His oxidation is generally catalyzed by trace metals that induce site-specific oxidation and can have drastic consequences on the pharmacokinetics or the activity of the protein, as observed for insulin and GH.

Phe and Tyr oxidation can yield Tyr oxidation products, which are electrophiles prone to 1,4- and 1,6-type addition: such modifications mediated aggregates formation in insulin and IFNβ-1a. Oxidation-induced aggregation of a monoclonal IgG, IFNα-2a, IFNα-2b and IFNβ-1a probably occurred via the same mechanism. All these oxidized and aggregated products were found to be more immunogenic than their native counterparts in mouse models, and for IFNα-2a also in human patients.

Trp oxidation, although occurring in the minority of the studied proteins, can also be responsible for bio-activity loss, as observed in some mAbs.

In conclusion, oxidation of peptides and proteins is an important degradation pathway. From the case studies discussed in this review, it is

clear that oxidation not only leads to changes in the primary structure, but also can perturb higher-order structures and induce aggregation, which in turn can have important biological consequences, such as altered pharmacokinetics, loss of function and enhanced immunogenicity. Therefore, it cannot be emphasized enough that control of oxidation during production, purification, formulation, transportation, storage and use of therapeutic proteins and peptides is of utmost importance for their quality, safety and efficacy.

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

[3] J. Mohammadian-Mosaabadi, H. Naderi-Manesh, N. Maghsoudi, R. Khalilzadeh, S. A. Shojaosadati, M. Ebrahimi. Effect of oxidative stress on the production of recombinant human interferon-gamma in Escherichia coli. *Biotechnol. Appl. Biochem.* **2005**, *41*, 37-42.

[4] R. Krishnamurthy, R. D. Madurawe, K. D. Bush, J. A. Lumpkin. Conditions Promoting Metal-Catalyzed Oxidations during Immobilized Cu-Iminodiacetic Acid Metal Affinity-Chromatography. *Biotechnol. Prog.* **1995**, *11*, 643-650.

[5] 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.

[6] T. Takenawa, A. Yokota, M. Oda, H. Takahashi, M. Iwakura. Protein oxidation during long storage: identification of the oxidation sites in dihydrofolate reductase from Escherichia coli through LC-MS and fragment studies. *J. Biochem.* **2009**, *145*, 517-523.

[7] C. Schöneich, L. B. Barrón. Posttranslational Oxidative Modifications of Proteins. *Encyclopedia of Analytical Chemistry.* **2006**.

[8] B. A. Kerwin, R. L. Remmele, Jr. Protect from light: photodegradation and protein biologics. *J. Pharm. Sci.* **2007**, *96*, 1468-1479.

[9] 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.

[10] R. Torosantucci, B. Kükrer, 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.

[11] K. C. Waterman, R. C. Adami, K. M. Alsante, J. Hong, M. S. Landis, F. Lombardo, C. J. Roberts. Stabilization of pharmaceuticals to oxidative degradation. *Pharm. Dev. Technol.* **2002**, *7*, 1-32.

[12] S. Li, C. Schöneich, R. T. Borchardt. Chemical instability of protein pharmaceuticals: Mechanisms of oxidation and strategies for stabilization. *Biotechnol. Bioeng.* **1995**, *48*, 490-500.

[13] E. R. Stadtman, B. S. Berlett. Fenton chemistry. Amino acid oxidation. *J. Biol. Chem.* **1991**, *266*, 17201-17211.

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

[15] E. R. Stadtman. Oxidation of free amino acids and amino acid residues in proteins by radiolysis and by metal-catalyzed reactions. *Annu. Rev. Biochem.* **1993**, *62*, 797-821.

[16] M. J. Davies. The oxidative environment and protein damage. *Biochim. Biophys. Acta* **2005**, *1703*, 93-109.

[17] S. Barelli, G. Canellini, L. Thadikkaran, D. Crettaz, M. Quadroni, J. S. Rossier, J. D. Tissot, N. Lion. Oxidation of proteins: Basic principles and perspectives for blood proteomics. *Proteomics Clin. Appl.* **2008**, *2*, 142-157.

[18] E. Shacter. Quantification and significance of protein oxidation in biological samples. *Drug Metab. Rev.* **2000**, *32*, 307-326.

[19] J. L. Cleland, M. F. Powell, S. J. Shire. The Development of Stable Protein Formulations - a Close Look at Protein Aggregation, Deamidation, and Oxidation. *Crit. Rev. Ther. Drug* **1993**, *10*, 307-377.

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

[21] K. R. Jayapal, K. F. Wlaschin, W. S. Hu, M. G. S. Yap. Recombinant protein therapeutics from CHO cells - 20 years and counting. *Chem. Eng. Prog.* **2007**, *103*, 40-47.

[22] T. Miyata, S. Takizawa, C. van Ypersele de Strihou. Hypoxia. 1. Intracellular sensors for oxygen and oxidative stress: novel therapeutic targets. *Am. J. Physiol. Cell. Physiol.* **2011**, *300*, C226-231.

[23] B. W. Row, R. G. Liu, W. Xu, L. Kheirandish, D. Gozal. Intermittent hypoxia is associated with oxidative stress and spatial learning deficits in the rat. *Am. J. Resp. Crit. Med.* **2003**, *167*, 1548-1553.

[24] V. Pialoux, R. Mounier. Hypoxia-induced oxidative stress in health disorders. *Oxid. Med. Cell. Longev.* **2012**, 940121.

[25] A. A. Lin, W. M. Miller. CHO cell responses to low oxygen: regulation of oxygen consumption and sensitization to oxidative stress. *Biotechnol. Bioeng.* **1992**, *40*, 505-516.

[26] B. X. Yan, Z. Yates, A. Balland, G. R. Kleemann. Human IgG1 Hinge Fragmentation as the Result of H2O2-mediated Radical Cleavage. *J. Biol. Chem.* **2009**, *284*, 35390-35402.

[27] Y. Wu, J. Levons, A. S. Narang, K. Raghavan, V. M. Rao. Reactive impurities in excipients: profiling, identification and mitigation of drug-excipient incompatibility. *AAPS Pharm. Sci. Tech.* **2011**, *12*, 1248-1263.

[28] E. T. Maggio. Polysorbates, peroxides, protein aggregation, and immunogenicity:a growing concern. *J. Excip. Food Chem.* **2012**, *Vol 3*.

[29] D. M. Miller, G. R. Buettner, S. D. Aust. Transition-Metals as Catalysts of Autoxidation Reactions. *Free Radic. Biol. Med.* **1990**, *8*, 95-108.

[30] X. Huang, C. S. Atwood, R. D. Moir, M. A. Hartshorn, R. E. Tanzi, A. I. Bush. Trace metal contamination initiates the apparent auto-aggregation, amyloidosis, and oligomerization of Alzheimer's Abeta peptides. *J. Biol. Inorg. Chem.* **2004**, *9*, 954-960.

[31] M. Lodovici, E. Bigagli. Oxidative stress and air pollution exposure. *J. Toxicol.*, **2011**, 487074.

[32] I. Romieu, F. Castro-Giner, N. Kunzli, J. Sunyer. Air pollution, oxidative stress and dietary supplementation: a review. *Eur. Respir. J.* **2008**, *31*, 179-197.

[33] G. R. Buettner, B. A. Jurkiewicz. Catalytic metals, ascorbate and free radicals: combinations to avoid. *Radiat. Res.* **1996**, *145*, 532-541.

[34] J. A. Ji, B. Y. Zhang, W. Cheng, Y. J. Wang. Methionine, Tryptophan, and Histidine Oxidation in a Model Protein, PTH: Mechanisms and Stabilization. *J. Pharm. Sci.* **2009**, *98*, 4485-4500.

[35] R. Rojas, G. Apodaca. Immunoglobulin transport across polarized epithelial cells. *Nat. Rev. Mol. Cell. Biol.* **2002**, *3*, 944-955.

[36] Z. An, G. Forrest, R. Moore, M. Cukan, P. Haytko, L. Huang, S. Vitelli, J. Z. Zhao, P. Lu, J. Hua, C. R. Gibson, B. R. Harvey, D. Montgomery, D. Zaller, F. Wang, W. Strohl. IgG2m4, an engineered antibody isotype with reduced Fc function. *MAbs* **2009**, *1*, 572-579.

[37] B. Al-Lazikani, A. M. Lesk, C. Chothia. Standard conformations for the canonical structures of immunoglobulins. *J. Mol. Biol.* **1997**, *273*, 927-948.

[38] H. Pan, K. Chen, L. Chu, F. Kinderman, I. Apostol, G. Huang. Methionine oxidation in human IgG2 Fc decreases binding affinities to protein A and FcRn. *Protein Sci.* **2009**, *18*, 424-433.

[39] G. M. Edelman, B. A. Cunningham, W. E. Gall, P. D. Gottlieb, U. Rutishauser, M. J. Waxdal. The covalent structure of an entire gammaG immunoglobulin molecule. *Proc. Natl. Acad. Sci. U. S. A.* **1969**, *63*, 78-85.

[40] U. Rutishauser, B. A. Cunningham, C. Bennett, W. H. Konigsberg, G. M. Edelman. Amino acid sequence of the Fc region of a human gamma G-immunoglobulin. *Proc. Natl. Acad. Sci. U. S. A.* **1968**, *61*, 1414-1421.

[41] E. van Loghem. Allotypic markers. *Monogr. Allergy.* **1986**, *19*, 40-51.

[42] H. K. Khor, M. E. Jacoby, T. C. Squier, G. C. Chu, D. Chelius. Identification of methionine sulfoxide diastereomers in immunoglobulin gamma antibodies using methionine sulfoxide reductase enzymes. *MAbs* **2010**, *2*, 299-308.

[43] R. G. Keck. The use of t-butyl hydroperoxide as a probe for methionine oxidation in proteins. *Anal. Biochem.* **1996**, *236*, 56-62.

[44] P. Qi, D. B. Volkin, H. Zhao, M. L. Nedved, R. Hughes, R. Bass, S. C. Yi, M. E. Panek, D. Wang, P. Dalmonte, M. D. Bond. Characterization of the photodegradation of a human IgG1 monoclonal antibody formulated as a high-concentration liquid dosage form. *J. Pharm. Sci.* **2009**, *98*, 3117-3130.

[45] H. Liu, G. Gaza-Bulseco, L. Zhou. Mass spectrometry analysis of photo-induced methionine oxidation of a recombinant human monoclonal antibody. *J. Am. Soc. Mass. Spectrom.* **2009**, *20*, 525-528.

[46] P. E. Rao, D. J. Kroon. Orthoclone OKT3. Chemical mechanisms and functional effects of degradation of a therapeutic monoclonal antibody. *Pharm. Biotechnol.* **1993**, *5*, 135-158.

[47] X. M. Lam, J. Y. Yang, J. L. Cleland. Antioxidants for prevention of methionine oxidation in recombinant monoclonal antibody HER2. *J. Pharm. Sci.* **1997**, *86*, 1250-1255.

[48] G. Gaza-Bulseco, S. Faldu, K. Hurkmans, C. Chumsae, H. Liu. Effect of methionine oxidation of a recombinant monoclonal antibody on the binding affinity to protein A and protein G. *J. Chromatogr. B. Analyt. Technol. Biomed. Life Sci.* **2008**, *870*, 55-62.

[49] A. Bertolotti-Ciarlet, W. R. Wang, R. Lownes, P. Pristatsky, Y. L. Fang, T. McKelvey, Y. Z. Li, Y. S. Li, J. Drummond, T. Prueksaritanont, J. Vlasak. Impact of methionine oxidation on the binding of human IgG1 to FcRn and Fc gamma receptors. *Mol. Immunol.* **2009**, *46*, 1878-1882.

[50] W. Wang, J. Vlasak, Y. Li, P. Pristatsky, Y. Fang, T. Pittman, J. Roman, Y. Wang, T. Prueksaritanont, R. Ionescu. Impact of methionine oxidation in human IgG1 Fc on serum half-life of monoclonal antibodies. *Mol. Immunol.* **2011**, *48*, 860-866.

[51] D. Liu, D. Ren, H. Huang, J. Dankberg, R. Rosenfeld, M. J. Cocco, L. Li, D. N. Brems, R. L. Remmele. Structure and stability changes of human IgG1 Fc as a consequence of methionine oxidation. *Biochemistry* **2008**, *47*, 5088-5100.

[52] S. Wang, R. Ionescu, N. Peekhaus, J. Y. Leung, S. Ha, J. Vlasak. Separation of post-translational modifications in monoclonal antibodies by exploiting subtle conformational changes under mildly acidic conditions. *J. Chromatogr. A.* **2010**, *1217*, 6496-6502.

[53] H. Liu, G. Gaza-Bulseco, T. Xiang, C. Chumsae. Structural effect of deglycosylation and methionine oxidation on a recombinant monoclonal antibody. *Mol. Immunol.* **2008**, *45*, 701-708.

[54] W. Burkitt, P. Domann, G. O'Connor. Conformational changes in oxidatively stressed monoclonal antibodies studied by hydrogen exchange mass spectrometry. *Protein. Sci.* **2010** , *19*, 826-835.

[55] D. Houde, Y. Peng, S. A. Berkowitz, J. R. Engen. Post-translational modifications differentially affect IgG1 conformation and receptor binding. *Mol. Cell. Proteomics* **2010**, *9*, 1716-1728.

[56] 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 .

[57] Q. Luo, M. K. Joubert, R. Stevenson, R. R. Ketchem, L. O. Narhi, J. Wypych. Chemical modifications in therapeutic protein aggregates generated under different stress conditions. *J. Biol. Chem.* **2011**, *286*, 25134-25144.

[58] M. K. Joubert, Q. Luo, Y. Nashed-Samuel, J. Wypych, L. O. Narhi. Classification and characterization of therapeutic antibody aggregates. *J. Biol. Chem.* **2011**, *286*, 25118-25133.

[59] M. Hensel, R. Steurer, J. Fichtl, C. Elger, F. Wedekind, A. Petzold, T. Schlothauer, M. Molhoj, D. Reusch, P. Bulau. Identification of potential sites for tryptophan oxidation in recombinant antibodies using tert-butylhydroperoxide and quantitative LC-MS. *PLoS One* **2011**, *6*, e17708.

[60] Z. P. Wei, J. H. Feng, H. Y. Lin, S. Mullapudi, E. Bishop, G. I. Tous, J. Casas-Finet, F. Hakki, R. Strouse, M. A. Schenerman. Identification of a single tryptophan residue as critical for binding activity in a humanized monoclonal antibody against respiratory syncytial virus. *Anal. Chem.* **2007**, *79*, 2797-2805.

[61] M. C. Gaudiano, M. Diociaiuti, P. Bertocchi, L. Valvo. Effects induced by hydroxyl radicals on salmon calcitonin: a RP-HPLC, CD and TEM study. *Biochim. Biophys. Acta.* **2003**, *1623*, 33-40.

[62] P. H. Tobler, F. A. Tschopp, M. A. Dambacher, W. Born, J. A. Fischer. Identification and characterization of calcitonin forms in plasma and urine of normal subjects and medullary carcinoma patients. *J. Clin. Endocrinol. Metab.* **1983**, *57*, 749-754.

[63] R. Neher, B. Riniker, R. Maier, P. G. Byfield, T. V. Gudmundsson, I. MacIntyre. Human calcitonin. *Nature* **1968**, *220*, 984-986.

[64] B. Riniker, R. Neher, R. Maier, F. W. Kahnt, P. G. Byfield, T. V. Gudmundsson, L. Galante, I. MacIntyre. Human calcitonin. I. Isolation and characterization. *Helv. Chim. Acta.* **1968**, *51*, 1738-1742.

[65] F. Mulinacci, E. Poirier, M. A. Capelle, R. Gurny, T. Arvinte. Enhanced physical stability of human calcitonin after methionine oxidation. *Eur. J. Pharm. Biopharm.* **2011**, *78*, 229-238.

[66] M. C. Gaudiano, M. Colone, C. Bombelli, P. Chistolini, L. Valvo, M. Diociaiuti. Early stages of salmon calcitonin aggregation: Effect induced by ageing and oxidation processes in water and in the presence of model membranes. *Biochim. Biophys. Acta* **2005**, *1750*, 134-145.

[67] V. Windisch, F. DeLuccia, L. Duhau, F. Herman, J. J. Mencel, S. Y. Tang, M. Vuilhorgne. Degradation pathways of salmon calcitonin in aqueous solution. *J. Pharm. Sci.* **1997**, *86*, 359-364.

[68] J. L. Reubsaet, J. H. Beijnen, A. Bult, E. Hop, S. D. Scholten, J. Teeuwsen, W. J. Underberg. Oxidation of recombinant methionyl human granulocyte colony stimulating factor. *J. Pharm. Biomed. Anal.* **1998**, *17*, 283-289.

[69] H. S. Lu, P. R. Fausset, L. O. Narhi, T. Horan, K. Shinagawa, G. Shimamoto, T. C. Boone. Chemical modification and site-directed mutagenesis of methionine residues in recombinant human granulocyte colony-stimulating factor: Effect on stability and biological activity. *Arch. Biochem. Biophys.* **1999**, *362*, 1-11.

[70] T. Arakawa, S. J. Prestrelski, L. O. Narhi, T. C. Boone, W. C. Kenney. Cysteine 17 of recombinant human granulocyte-colony stimulating factor is partially solvent-exposed. *J. Protein. Chem.* **1993**, *12*, 525-531.

[71] S. W. Raso, J. Abel, J. M. Barnes, K. M. Maloney, G. Pipes, M. J. Treuheit, J. King, D. N. Brems. Aggregation of granulocyte-colony stimulating factor in vitro involves a conformationally altered monomeric state. *Protein. Sci.* **2005**, *14*, 2246-2257.

[72] F. Mulinacci, S. E. J. Bell, M. A. H. Capelle, R. Gurny, T. Arvinte. Oxidized Recombinant Human Growth Hormone That Maintains Conformational Integrity. *J. Pharm. Sci.* **2011**, *100*, 110-122.

[73] L. C. Teh, L. J. Murphy, N. L. Huq, A. S. Surus, H. G. Friesen, L. Lazarus, G. E. Chapman. Methionine oxidation in human growth hormone and human chorionic somatomammotropin. Effects on receptor binding and biological activities. *J. Biol. Chem.* **1987**, *262*, 6472-6477.

[74] F. Mulinacci, M. A. Capelle, R. Gurny, A. F. Drake, T. Arvinte. Stability of human growth hormone: influence of methionine oxidation on thermal folding. *J. Pharm. Sci.* **2011**, *100*, 451-463.

[75] B. C. Cunningham, J. A. Wells. Comparison of a structural and a functional epitope. *J. Mol. Biol.* **1993**, *234*, 554-563.

[76] A. M. de Vos, M. Ultsch, A. A. Kossiakoff. Human growth hormone and extracellular domain of its receptor: crystal structure of the complex. *Science* **1992**, *255*, 306-312.

[77] F. Hepner, E. Cszasar, E. Roitinger, G. Lubec. Mass spectrometrical analysis of recombinant human growth hormone (Genotropin(R)) reveals amino acid substitutions in 2% of the expressed protein. *Proteome Sci.* **2005**, *3*, 1.

[78] D. Steinmann, J. A. Ji, Y. J. Wang, C. Schöneich. Oxidation of human growth hormone by oxygen-centered radicals: formation of Leu-101 hydroperoxide and Tyr-103 oxidation products. *Mol. Pharm.* **2012**, *9*, 803-814.

[79] S. H. Chang, G. M. Teshima, T. Milby, B. GilleceCastro, E. CanovaDavis. Metal-catalyzed photooxidation of histidine in human growth hormone. *Anal. Biochem.* **1997**, *244*, 221-227.

[80] S. W. Hovorka, J. Hong, J. L. Cleland, C. Schöneich. Metal-catalyzed oxidation of human growth hormone: modulation by solvent-induced changes of protein conformation. *J. Pharm. Sci.* **2001**, *90*, 58-69.

[81] F. Zhao, E. Ghezzo-Schöneich, G. I. Aced, J. Hong, T. Milby, C. Schöneich. Metal-catalyzed oxidation of histidine in human growth hormone. Mechanism, isotope effects, and inhibition by a mild denaturing alcohol. *J. Biol. Chem.* **1997**, *272*, 9019-9029.

[82] F. Sanger. Fractionation of oxidized insulin. *Biochem. J.* **1949**, *44*, 126-128.

[83] H. R. Costantino, R. Langer, A. M. Klibanov. Moisture-Induced Aggregation of Lyophilized Insulin. *Pharm. Res.* **1994**, *11*, 21-29.

[84] S. W. Hovorka, H. Biesiada, T. D. Williams, A. Huhmer, C. Schöneich. High sensitivity of Zn2+ insulin to metal-catalyzed oxidation: detection of 2-oxo-histidine by tandem mass spectrometry. *Pharm. Res.* **2002**, *19*, 530-537.

[85] V. Sadineni, C. Schöneich. Selective oxidation of Zn2+ Insulin catalyzed by Cu2+. *J. Pharm. Sci.* **2007**, *96*, 1844-1847.

[86] R. Tantipolphan, S. Romeijn, J. den Engelsman, R. Torosantucci, T. Rasmussen, W. Jiskoot. Elution behavior of insulin on high-performance size exclusion chromatography at neutral pH. *J. Pharm. Biomed. Anal.* **2010**, *52*, 195-202.

[87] I. Jonassen, S. Havelund, T. Hoeg-Jensen, D. B. Steensgaard, P. O. Wahlund, U. Ribel. Design of the novel protraction mechanism of insulin degludec, an ultra-long-acting basal insulin. *Pharm. Res.* **2012**, *29*, 2104-2114.

[88] A. C. Maritim, R. A. Sanders, J. B. Watkins, 3rd. Diabetes, oxidative stress, and antioxidants: a review. *J. Biochem. Mol. Toxicol.* **2003**, *17*, 24-38.

[89] S. J. Hunter, A. C. Boyd, F. P. O'Harte, A. M. McKillop, M. I. Wiggam, M. H. Mooney, J. T. McCluskey, J. R. Lindsay, C. N. Ennis, R. Gamble, B. Sheridan, C. R. Barnett, H. McNulty, P. M. Bell, P. R. Flatt. Demonstration of glycated insulin in human diabetic plasma and decreased biological activity assessed by euglycemic-hyperinsulinemic clamp technique in humans. *Diabetes* **2003**, *52*, 492-498.

[90] P. J. Thornalley, A. Langborg, H. S. Minhas. Formation of glyoxal, methylglyoxal and 3-deoxyglucosone in the glycation of proteins by glucose. *Biochem. J.* **1999**, *344 Pt 1*, 109-116.

[91] X. Jia, D. J. Olson, A. R. Ross, L. Wu. Structural and functional changes in human insulin induced by methylglyoxal. *FASEB J.* **2006**, *20*, 1555-1557.

[92] N. J. Pillon, R. E. Vella, L. Souleere, M. Becchi, M. Lagarde, C. O. Soulage. Structural and functional changes in human insulin induced by the lipid peroxidation byproducts 4-hydroxy-2-nonenal and 4-hydroxy-2-hexenal. *Chem. Res. Toxicol.* **2011**, *24*, 752-762.

[93] R. Z. Cheng, S. Kawakishi. Site-specific oxidation of histidine residues in glycated insulin mediated by Cu2+. *Eur. J. Biochem.* **1994**, *223*, 759-764.

[94] S. Guedes, R. Vitorino, M. R. M. Domingues, F. Amado, P. Domingues. Oxidative modifications in glycated insulin. *Anal. Bioanal. Chem.* **2010**, *397*, 1985-1995.

[95] J. Gliemann, S. Gammeltoft. The biological activity and the binding affinity of modified insulins determined on isolated rat fat cells. *Diabetologia* **1974**, *10*, 105-113.

[96] D. H. Montes-Cortes, J. J. Hicks, G. M. Ceballos-Reyes, J. R. Garcia-Sanchez, R. Medina-Navarro, I. M. Olivares-Corichi. Chemical and functional changes of human insulin by in

vitro incubation with blood from diabetic patients in oxidative stress. *Metabolism* **2010**, *59*, 935-942.

[97] I. M. Olivares-Corichi, G. Ceballos, R. Medina-Santillan, R. Medina-Navarro, A. M. Guzman-Grenfell, J. J. Hicks. Oxidation by reactive oxygen species (ROS) alters the structure of human insulin and decreases the insulin-dependent D-glucose-C14 utilization by human adipose tissue. *Front. Biosci.* **2005**, *10*, 3127-3131.

[98] V. K. Sharma, D. S. Kalonia. Polyethylene glycol-induced precipitation of interferon alpha-2a followed by vacuum drying: development of a novel process for obtaining a dry, stable powder. *AAPS Pharm. Sci.* **2004**, *6*, E4.

[99] L. Larocque, A. Bliu, R. Xu, A. Diress, J. Wang, R. Lin, R. He, M. Girard, X. Li. Bioactivity determination of native and variant forms of therapeutic interferons. *J. Biomed. Biotechnol.* **2011**, *2011*, 174615.

[100] J. C. Ryff. Clinical investigation of the immunogenicity of interferon-alpha 2a. *J. Interferon Cytokine Res.* **1997**, *17 Suppl 1*, S29-33.

[101] E. Hochuli. Interferon immunogenicity: technical evaluation of interferon-alpha 2a. *J. Interferon Cytokine Res.* **1997**, *17 Suppl 1*, S15-21.

[102] M. Cindric, N. Galic, M. Vuletic, M. Klaric, V. Drevenkar. Evaluation of recombinant human interferon alpha-2b structure and stability by in-gel tryptic digestion, H/D exchange and mass spectrometry. *J. Pharm. Biomed. Anal.* **2006**, *40*, 781-787.

[103] G. L. Praveen Kumar RB, Jinghui Shen, Kim Gaspar, John Docherty, Marianna Foldvari. Stabilization of Interferon alpha-2b in a Topical Cream. *Pharmaceutical Technology* **2009**, *33*, 80-86.

[104] G. Gitlin, A. Tsarbopoulos, S. T. Patel, W. Sydor, B. N. Pramanik, S. Jacobs, L. Westreich, S. Mittelman, J. N. Bausch. Isolation and characterization of a monomethioninesulfoxide variant of interferon alpha-2b. *Pharm. Res.* **1996**, *13*, 762-769.

[105] 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.

[106] S. Orru, A. Amoresano, R. Siciliano, R. Napoleoni, O. Finocchiaro, A. Datola, E. De Luca, A. Sirna, P. Pucci. Structural analysis of modified forms of recombinant IFN-beta produced under stress-simulating conditions. *Biol. Chem.* **2000**, *381*, 7-17.

[107] M. Karpusas, M. Nolte, C. B. Benton, W. Meier, W. N. Lipscomb, S. Goelz. The crystal structure of human interferon beta at 2.2-angstrom resolution. *Proc. Natl. Acad. Sci. U. S. A.* **1997**, *94*, 11813-11818.

[108] L. Runkel, W. Meier, R. B. Pepinsky, M. Karpusas, A. Whitty, K. Kimball, M. Brickelmaier, C. Muldowney, W. Jones, S. E. Goelz. Structural and functional differences between glycosylated and non-glycosylated forms of human interferon-beta (IFN-beta). *Pharm. Res.* **1998**, *15*, 641-649.

[109] 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.* **2011**, *28*, 2393-2402.

[110] R. Torosantucci, V. S. Sharov, M. van Beers, V. Brinks, C. Schöneich, W. Jiskoot. Identification of Oxidation Sites and Covalent Cross-Links in Metal Catalyzed Oxidized Interferon Beta-1a: Potential Implications for Protein Aggregation and Immunogenicity. *Mol. Pharm.* **2013**, *10,* 2311-2322.

[111] C. Avanti, J. P. Amorij, D. Setyaningsih, A. Hawe, W. Jiskoot, J. Visser, A. Kedrov, A. J. M. Driessen, W. L. J. Hinrichs, H. W. Frijlink. A New Strategy to Stabilize Oxytocin in Aqueous Solutions: I. The Effects of Divalent Metal Ions and Citrate Buffer. *Aaps Journal* **2011**, *13*, 284-290.

[112] A. Hawe, R. Poole, S. Romeijn, P. Kasper, R. van der Heijden, W. Jiskoot. Towards heat-stable oxytocin formulations: analysis of degradation kinetics and identification of degradation products. *Pharm. Res.* **2009**, *26*, 1679-1688.

[113] R. C. M. A. Rosei, C. Blarzino, C. Foppoli, L. Mosca. The oxidation of oxytocin and vasopressin by peroxidase/H2O2 system. *Amino Acids* **1995**, *8*, 385-391.

[114] Y. Kato, N. Kitamoto, Y. Kawai, T. Osawa. The hydrogen peroxide/copper ion system, but not other metal-catalyzed oxidation systems, produces protein-bound dityrosine. *Free Radic. Biol. Med.* **2001**, *31*, 624-632.

[115] B. Hocher, F. P. Armbruster, S. Stoeva, C. Reichetzeder, H. J. Gron, I. Lieker, D. Khadzhynov, T. Slowinski, H. J. Roth. Measuring parathyroid hormone (PTH) in patients with oxidative stress--do we need a fourth generation parathyroid hormone assay? *PLoS One* **2012**, *7*, e40242.

[116] M. Kamberi, Y. J. Kim, B. Jun, C. M. Riley. The effects of sucrose on stability of human brain natriuretic peptide [hBNP (1-32)] and human parathyroid hormone [hPTH (1-34)]. *J. Pept. Res.* **2005**, *66*, 348-356.

[117] J. A. Barden, B. E. Kemp. NMR solution structure of human parathyroid hormone(1-34). *Biochemistry* **1993**, *32*, 7126-7132.

[118] J. E. Zull, S. K. Smith, R. Wiltshire. Effect of methionine oxidation and deletion of amino-terminal residues on the conformation of parathyroid hormone. Circular dichroism studies. *J. Biol. Chem.* **1990**, *265*, 5671-5676.

[119] Y. Nabuchi, E. Fujiwara, K. Ueno, H. Kuboniwa, Y. Asoh, H. Ushio. Oxidation of recombinant human parathyroid hormone: Effect of oxidized position on the biological activity. *Pharm. Res.* **1995**, *12*, 2049-2052.

[120] O. Mozziconacci, J. A. Ji, Y. J. Wang, C. Schöneich. Metal-catalyzed oxidation of protein methionine residues in human parathyroid hormone (1-34): formation of homocysteine and a novel methionine-dependent hydrolysis reaction. *Mol. Pharm.* **2013**, *10*, 739-755.

[121] A. L. Frelinger, 3rd, J. E. Zull. Oxidized forms of parathyroid hormone with biological activity. Separation and characterization of hormone forms oxidized at methionine 8 and methionine 18. *J. Biol. Chem.* **1984**, *259*, 5507-5513.

[122] J. S. Woodhead, J. L. O'Riordan, H. T. Keutmann, M. L. Stoltz, B. F. Dawson, H. D. Niall, C. J. Robinson, J. T. Potts, Jr. Isolation and chemical properties of porcine parathyroid hormone. *Biochemistry* **1971**, *10*, 2787-2792.

[123] M. P. Caulfield, R. L. McKee, M. E. Goldman, L. T. Duong, J. E. Fisher, C. T. Gay, P. A. DeHaven, J. J. Levy, E. Roubini, R. F. Nutt, et al. The bovine renal parathyroid hormone (PTH) receptor has equal affinity for two different amino acid sequences: the receptor binding domains of PTH and PTH-related protein are located within the 14-34 region. *Endocrinology* **1990**, *127*, 83-87.

[124] A. L. Frelinger, 3rd, J. E. Zull. The role of the methionine residues in the structure and function of parathyroid hormone. *Arch. Biochem. Biophys.* **1986**, *244*, 641-649.