Amino Acids (2019) 51:1409–1431
https://doi.org/10.1007/s00726-019-02787-2

REVIEW ARTICLE

Susceptibility of protein therapeutics to spontaneous chemical modifications by oxidation, cyclization, and elimination reactions

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Received: 12 May 2019 / Accepted: 7 September 2019 / Published online: 1 October 2019

Abstract

Peptides and proteins are preponderantly emerging in the drug market, as shown by the increasing number of biopharmaceutics already approved or under development. Biomolecules like recombinant monoclonal antibodies have high therapeutic efficacy and offer a valuable alternative to small-molecule drugs. However, due to their complex three-dimensional structure and the presence of many functional groups, the occurrence of spontaneous conformational and chemical changes is much higher for peptides and proteins than for small molecules. The characterization of biotherapeutics with modern and sophisticated analytical methods has revealed the presence of contaminants that mainly arise from oxidation- and elimination-prone amino-acid side chains. This review focuses on protein chemical modifications that may take place during storage due to (1) oxidation (methionine, cysteine, histidine, tyrosine, tryptophan, and phenylalanine), (2) intra- and inter-residue cyclization (aspartic and glutamic acid, asparagine, glutamine, N-terminal dipeptidyl motifs), and (3) β-elimination (serine, threonine, cysteine, cystine) reactions. It also includes some examples of the impact of such modifications on protein structure and function.

Keywords Amino acid · Spontaneous posttranslational modification · Oxidation · Cyclization · Elimination · Protein degradation

Introduction

The market value of peptide and protein drugs has been experiencing a significant expansion, as reflected by the increasing number of approved synthetic peptides and recombinant monoclonal antibodies (mAbs) for the therapy of different types of human health disorders and diseases (Ecker et al. 2015; Lau and Dunn 2018). To this regard, the introduction of a drug into the market implies that all the regulatory requirements in terms of safety and quality have been addressed. In the case of peptides and proteins, which are medium-to-large molecules with high susceptibility to a number of spontaneous or enzyme-dependent chemical modifications, the full analytical characterization of the final product as well as the efficient implementation of the so-called quality-by-design concept (Juran 1992) in the manufacturing process are a big challenge.

Enzyme-catalyzed posttranslational modifications (PTMs) of proteins are used by Nature to expand the chemical space beyond the limit set by the 20 encoded amino acids. However, the presence of PTMs (i.e., glycosylation) in recombinant and isolated proteins gives rise to heterogeneous preparations of biotherapeutics. In addition, spontaneous chemical changes may occur due to the inherent reactivity of peptides and proteins. Therefore, the detection and characterization of any modification at the amino-acid side chain and backbone level is crucial for both regulatory compliance and reliability of the drug potency assessment (Wang et al. 2007). To date, the most powerful technique to assess the chemical identity of biotherapeutics is mass spectrometry (MS): for example, 79 out of the 80 biologics license applications electronically submitted to the FDA in the period 2000–2015 provide quality attributes determined by MS, the following being found in more than 50% of the applications:

Handling Editor: J. D. Wade.

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amino-acid sequence, molecular mass, disulfide bonds, glycosylation, N-/C-terminal sequence variants, deamidation, and oxidation (Rogstad et al. 2017).

The most common MS workflows rely on three types of approaches that utilize MS alone or hyphenated MS, like liquid chromatography–MS (LC–MS) or LC–MS/MS (Beck et al. 2013; Berkowitz et al. 2012; Terral et al. 2016; Zhang et al. 2014): (1) top-down (MS fragmentation of the intact protein under native or denaturing conditions), (2) middle-down (MS fragmentation of subunits), and (3) bottom-up (MS fragmentation of the peptides obtained by protein digestion) (Lermyte et al. 2019). Each workflow presents advantages and disadvantages: for example, the top-down workflow does not require long protocols to prepare the MS sample, but it suffers from inefficient fragmentation due to the large size of the proteins, especially of mAbs with molecular mass close to 150 kDa. This results in incomplete amino-acid sequence coverage, despite the use of sophisticated MS methods such as electron-transfer dissociation (ETD) with quadrupole time-of-flight (qTOF) or orbitrap high-resolution MS (Fornelli et al. 2012; Tsybin et al. 2011), electron-capture dissociation (ECD) with electrospray ionization (ESI) Fourier-transform ion cyclotron resonance (FTICR) MS (Mao et al. 2013), ESI-FTICR-MS coupled to electrochemistry for the online reduction of disulfide bonds (Nicolardi et al. 2014), matrix-assisted-laser-desorption-ionization (MALDI) in-source decay (ISD) with FTICR-MS (van der Burgt et al. 2019), combined ECD, and collisionally activated dissociation (CAD) with FTICR-MS (Jin et al. 2019). The middle-down MS workflow allows increasing the sequence coverage significantly, but it requires the partial proteolysis and deglycosylation of the protein: in the case of mAbs, the enzymes IdeS (immunoglobulin-degrading enzyme of *Streptococcus pyogenes*) (von Pawel-Rammingen et al. 2002) and GingisKHAN™ (recombinant gingipain K from *Porphyromonas gingivalis* (Okamoto et al. 1996; Scott et al. 1993), commercialized by Genovis AB, Lund, Sweden), which cleave the heavy chain, respectively, below or above the hinge region, as well as glycosidases and sialidases are used to obtain large subunits that are then analyzed under denaturing (Jin et al. 2019; van der Burgt et al. 2019) or native conditions (Wohlschlager et al. 2018). The bottom-up MS workflow is routinely used for sequence determination, but it requires long sample preparation, with the risk of introducing modifications, for example during trypsin digestion, like additional deamidation of asparagine-glycine (Asn-Gly) motifs and of N-terminal glutamine (Gln) (Bongers et al. 2000; Krokhin et al. 2006).

Several spontaneous PTMs have been characterized by bottom-up and middle-down protocols: for example, scrambled disulfide bonds in heat-stressed humanized mAb HER2 (human epidermal growth factor receptor 2) have been found by analyzing the tryptic and Lys-C-digested peptides with LC-ETD/CAD-MS (Wang et al. 2011a). Methionine (Met) oxidation of three different biotherapeutics treated with H_2O_2 (the mAbs rituximab and adalimumab, and the IgG1-Fc fusion protein etanercept) has been detected and quantified by applying a middle-down approach with LC–MS analysis (Regl et al. 2017). Furthermore, a systematic study on the stability of IgG1-Fc glycoforms containing 8–12, 5, or no mannose units under various stress conditions (photo-irradiation, oxidation with H_2O_2, $CuCl_2$/ascorbate, or a free-radical initiator, and long storage at 40 °C) has shown by a bottom-up approach that different degradation profiles are obtained, depending on the mannose content and the stress condition: for example, a deamidation product (already detectable in the control) has been found to accumulate faster in the absence than in the presence of mannose units at 40 °C (Mozziconacci et al. 2016a). Surprisingly, also a tryptophan (Trp) degradation product (glycine hydroperoxide) was already detectable in the control, but after 1 month at 40 °C, it was more abundant in the presence than in the absence of mannose units. Regarding the oxidation stress, the highest amount of oxidized Met was obtained with H_2O_2, but only for the high-mannose and no mannose glycoforms, clearly showing that both the stress protocol and the type of glycan may strongly affect the formation of impurities (Mozziconacci et al. 2016a).

It is well recognized that biotherapeutics carry the intrinsic risk of being immunogenic (Büttel et al. 2011; Pineda et al. 2016), a characteristic that is the likely consequence of multiple factors (type of production, impurities, and mode of administration of the drug, as well as patient's conditions) (Pineda et al. 2016) and, thus, difficult to control or even to suppress. Nevertheless, it would be very important, to monitor the drug immunogenicity not only before but also after drug approval, as shown, for example, by the case of the two mAbs adalimumab (Bartelds et al. 2011; Bender et al. 2007) and infliximab (Pascual-Salcedo et al. 2011): rheumatoid arthritis patients long-treated with these two drugs have been found to produce anti-drug Abs negatively affecting the drug response and efficacy. However, immunogenic responses of patients have been reported for several other approved mAbs (Baker et al. 2010; Kuriakose et al. 2016).

As mentioned above, drug impurities may increase the risk of undesired immunogenicity: these also include protein aggregates and PTMs (Baker et al. 2010; Büttel et al. 2011; Krishna and Nadler 2016; Kuriakose et al. 2016). The relationship between PTMs and immunogenic events is well known, although this is especially investigated in the area of autoimmune diseases (e.g., citrullination of myelic basic protein in rheumatoid arthritis, oxidation of insulin in type I diabetes, and deamidation of transglutaminase in celiac disease) (Doyle and Mamula 2012; Eggleton et al. 2008). PTMs may induce changes of the protein structure (Mallagaray et al. 2019), but they might also modify the type of

antigens generated (Krishna and Nadler 2016; Kuriakose et al. 2016): for example, the presence of an iso-aspartyl (iso-Asp) residue as deamidation product of cytochrome *c* has led to a different digestion profile with cathepsin D compared to the native protein (Doyle et al. 2007). In addition, mice immunized with an iso-Asp-containing cytochrome *c* peptide antigen developed Abs cross-reacting with both the iso-Asp-containing peptide and the native protein (Mamula et al. 1999). Two other examples are related to the oxidation of the collagen-like complement component C1q and collagen type II: in the first one, animal immunization with C1q treated with H_2O_2 generated Abs cross-reacting with unmodified C1q or with collagen type II, suggesting a role of C1q oxidation (e.g., by reactive oxygen species) in the induction of arthritis (Trinder et al. 1997). In the second one, a collagen type II peptide antigen containing a galactosylated hydroxylysine, as product of lysine oxidation followed by O-glycosylation, has been proposed to be immunodominant in collagen-induced arthritis (Corthay et al. 1998).

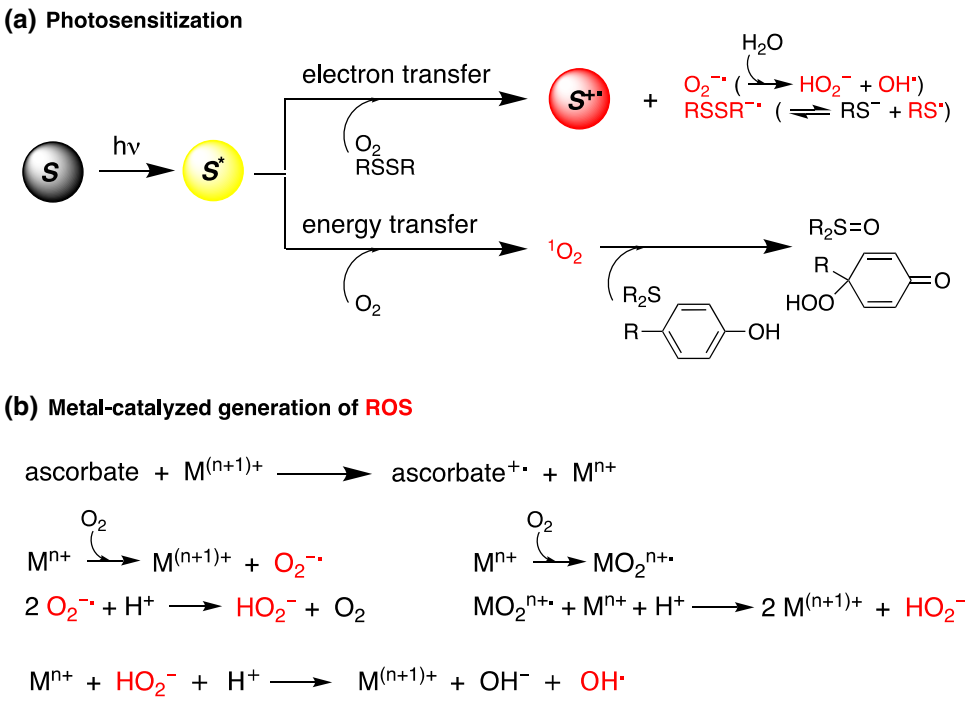
All together, these findings clearly indicate the implication of spontaneous PTMs in protein immunogenicity. In the case of biotherapeutics, while it is not possible to avoid their chemical modification in vivo, it is at least possible to control the presence of PTMs in the drug formulations thanks to the impressive advances made especially in MS, as briefly mentioned above.

Being the chemical space of spontaneous modifications of peptides and proteins very broad, only the most common by-products from three types of reactions, which may occur during storage, will be reviewed in the following sections: (1) oxidation of Met, cysteine (Cys), histidine (His), tyrosine (Tyr), Trp and phenylalanine (Phe), (2) intra- and inter-residue cyclization involving aspartate (Asp), Asn, glutamate (Glu), Gln and N-terminal dipeptidyl motifs, (3) β-elimination of serine (Ser), threonine (Thr), Cys, and cystine.

Oxidation of sulfur-containing and aromatic amino acids

Mechanisms generating reactive oxygen species (ROS)

Among the 20 natural amino acids, the sulfur-containing Met and Cys, as well as the aromatic His, Trp, Tyr, and Phe contain the most oxidation-prone side chains (Davies and Truscott 2001). Oxidation may arise from photosensitization processes that can be distinguished in direct (type I) and indirect (type II) photo-oxidation, depending on whether the oxidation of the target occurs via direct absorption of light followed by electron transfer, or through interaction with singlet oxygen ($^1O_2$) (Scheme 1a). In the first mechanism, direct interaction of an endogenous chromophore (e.g., an aromatic moiety) with UV light results in the production of excited states, followed by electron transfer to suitable acceptors such as molecular oxygen (O_2) or disulfides (Benasson et al. 1983) to give the corresponding radical cations and reactive oxygen species (ROS), including the superoxide radical anion ($O_2^{\cdot-}$) and its disproportionation products hydrogen



Scheme 1 Oxidation processes generating reactive oxygen species (ROS) by **a** photosensitization and **b** metal catalysis

peroxide (H_2O_2) and hydroxyl radical ($OH^{\cdot}$) (Davies and Gilbert 1992), or disulfide radical anions in equilibrium with thiolates and thiyl radicals (Schöneich 2012, 2016).

The second mechanism, by which photo-oxidation may occur in proteins, involves indirect energy transfer to O_2 from a photosensitizer, which generates $^1O_2$ in situ (Scheme 1a). This reacts with sulfides and aromatic residues of proteins, producing sulfoxides, hydroperoxides, and other derivatives (Davies and Truscott 2001; Ghogare and Greer 2016).

Besides photosensitization processes, transition metals, such as Fe(II) or Cu(I), have been also widely recognized as efficient catalysts for oxidative processes in proteins, which are referred to as metal-catalyzed oxidations (MCOs). The latter involve either direct metal-induced oxidation of the target, or, most commonly, the reaction of the target with ROS that are produced by transition-metal complexation of molecular oxygen in the presence of an appropriate electron donor (e.g., ascorbate) (Scheme 1b) (Li et al. 1995).

Oxidation processes may occur during production, purification, and storage, leading to the conversion of oxidation-prone residues to the corresponding oxidized species (Table 1), and, eventually, to substantial alteration of the protein stability and folding (Torosantucci et al. 2014).

Mechanisms of methionine oxidation

Oxidation of solvent-exposed Met residues in proteins has long being object of study, and significant efforts have been made to gain deeper insights into the mechanistic features of this reaction as well as the biological consequences resulting therefrom (Torosantucci et al. 2014). Formally, Met can undergo oxidation through a double- (by non-radical oxidants such as peroxides or hypochlorous acid) or single-electron transfer (e.g., by metal catalysis or photo-irradiation) (Schöneich 2005). In the presence of H_2O_2, the double-electron oxidation occurs at the sulfur atom producing the corresponding diastereomeric sulfoxide products in equal amounts (Sharov et al. 1999), which may further oxidize to sulfones under strong oxidizing conditions (Garner et al. 1998; Schöneich 2005) (Scheme 2a). The reaction follows a pH-independent mechanism involving a nucleophilic attack of the Met sulfide on the oxygen atom of the oxidant to form a water-stabilized intermediate, followed by proton transfer (Chu et al. 2004).

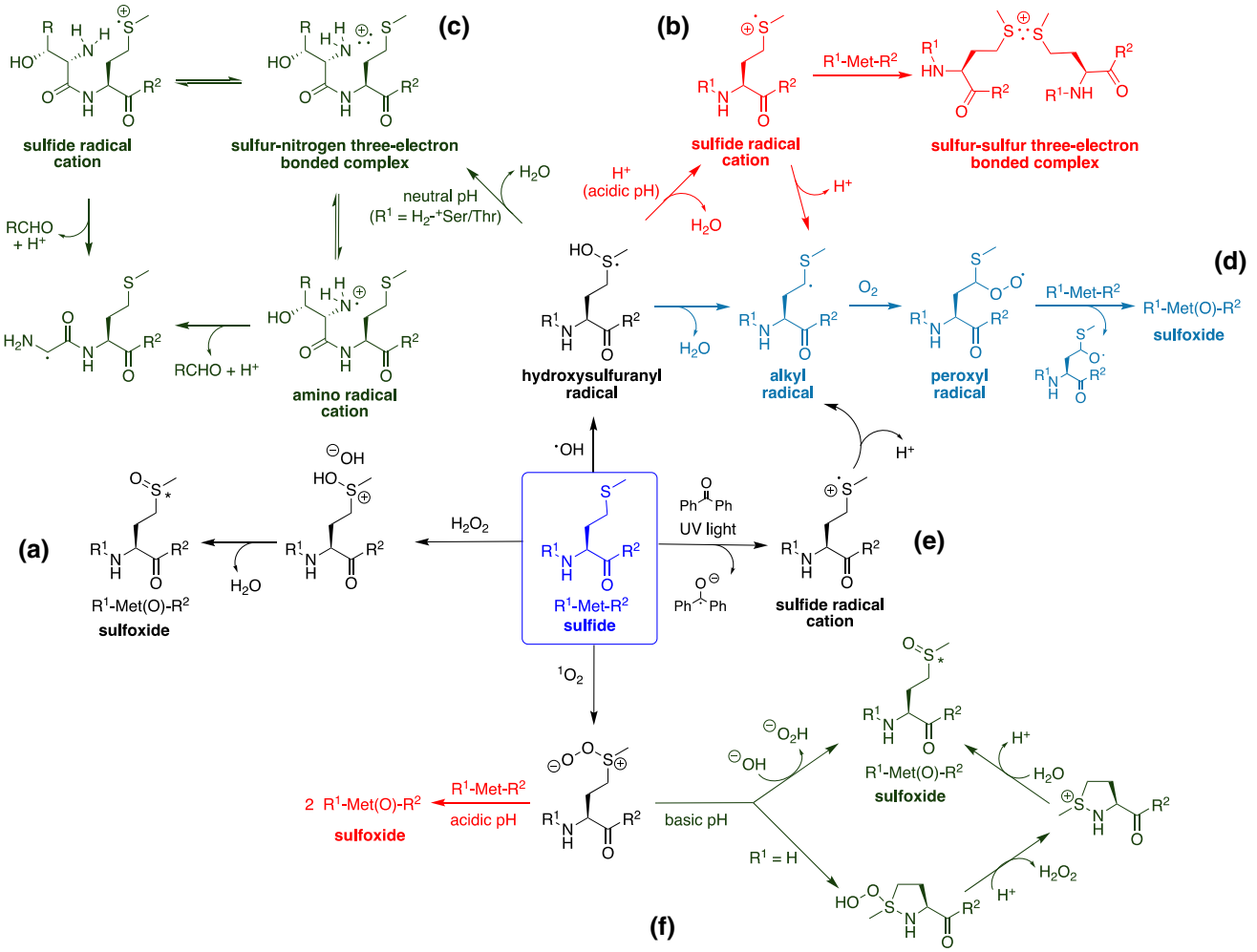
Met oxidation may also involve the hydroxyl radical $OH^{\cdot}$, which affords different products depending on pH, substrate concentration, and/or neighboring groups. It has been shown that addition of $OH^{\cdot}$ occurs predominantly at the sulfur atom to yield a hydroxysulfuranyl radical (Bonifacic et al. 2000). The latter may be protonated at acidic pH and eliminate a water molecule, generating the corresponding sulfide radical cation that is stabilized by association with a non-oxidized Met residue in a sulfur–sulfur three-electron-bonded complex (Schöneich 2005; Yashiro et al. 2005) (Scheme 2b). An intramolecular sulfur-amide oxygen three-electron bonded complex can also be formed during the Fenton reaction of the Met-His sequence, which leads to hydrolysis of the Met-His peptide bond (Mozziconacci et al. 2013, 2016b). At neutral pH, the hydroxysulfuranyl radical may undergo an intramolecular acid–base reaction with a protonated amino group, which leads to water elimination and a stabilized sulfur-nitrogen three-electron-bonded complex. However, this species is in equilibrium with sulfide or amino-radical cations that may decompose via heterolytic cleavage of neighboring bonds (Scheme 2c) (Schöneich et al. 1994).

Besides water elimination by protonation of the OH group, the hydroxysulfuranyl radical may eliminate water by hydrogen abstraction from neighboring alkyl groups, generating alkyl radicals that can be converted into peroxyl radicals by reaction with O_2. These, in turn, can oxidize a second Met residue to sulfoxide (Schöneich et al. 1993) (Scheme 2d). Alkyl radicals of Met residues can be built also by the corresponding sulfide radical cation upon proton abstraction (Schöneich et al. 1993).

Generation of the Met sulfide radical cation can also be triggered by light (Scheme 2e). For example, in a type I photo-oxidation, electron transfer from the sulfur atom to a triplet sensitizer like benzophenone or 4-carboxybenzophenone occurs, leading to the sulfide radical cation that can additionally produce Met alkyl radicals by proton abstraction (Pedzinski et al. 2009). Type II oxidation has also been reported (Scheme 2f): in this case, the reaction with $^1O_2$ builds a zwitterion intermediate that may either oxidize a second Met residue with the production of two Met(O) at acidic pH, or be converted into Met(O) with formation of

Table 1 Common oxidation products of oxidation-prone amino acids

Residue	Oxidation product
Met	Sulfoxide, sulfone
Cys	Sulfinic and sulfonic acid, di- and trisulfide
His	2-oxo-His, cross-linked adducts
Trp	Hydroxy-Trp, kynurenine (Kyn), 3-hydroxy-Kyn, N-formyl-kynurenine (NFK), 3-hydroxy-NFK
Tyr	3,4-Dihydroxyphenylalanine (DOPA), 2-amino-3-(3,4-dioxocyclohexa-1,5-dien-1-yl) propanoic acid (DOCH)



Scheme 2 Possible oxidation processes of Met. **a** Double-electron and **b**–**d** single-electron transfer as well as **e**, **f** photo-oxidation mechanisms are shown

H_2O_2 at basic pH by an intramolecular or intermolecular mechanism (Sysak et al. 1977).

Susceptibility of mAbs to methionine oxidation

Met oxidation in protein therapeutics has been correlated with a number of adverse effects, including loss of function (Hsu et al. 1996), decrease in folding stability (Mulinacci et al. 2011), as well as increase in aggregation propensity (Mulinacci et al. 2013). Met oxidation in vitro is generally obtained by treatment with oxidizing agents such as H_2O_2 (Li et al. 1995; Regl et al. 2017), *t*-butylhydroperoxide (*t*BHP) (Li et al. 1995), or with UV light irradiation (Lam et al. 1997; Wei et al. 2007). However, the susceptibility of a Met residue to oxidation significantly depends on its location within the protein structure. For example, when subjecting the recombinant IgG1 antibody HER2 to a 20 h treatment with *t*BHP, light-chain Met-4 and heavy-chain Met-107 and Met-361 were only slightly oxidized, whereas the two solvent-exposed heavy-chain Met-255 and Met-431, both located in the Fc region, underwent significant oxidation (Shen et al. 1996). Interestingly, when a fully human recombinant IgG1 monoclonal antibody was incubated in formulation buffer for 12 months at 25 °C, the preferential oxidation of Met-255 and Met-431 occurred only on one heavy chain, differently from a chemically stressed sample subjected to *t*BHP treatment, which was oxidized at both heavy chains (Chumsae et al. 2007).

Due to the structural perturbation induced, oxidation of Met-255 and Met-431 has been associated with altered thermal stability as well as to aggregation and deamidation propensity of the Fc part (Liu et al. 2008). In addition, decreased binding affinity for protein A, protein G and neonatal Fc receptor (FcR_n) has been observed (Bertolotti-Ciarlet et al. 2009; Gaza-Bulseco et al. 2008; Pan et al. 2009), together with a reduction of the serum half-life for highly oxidized species (Wang et al. 2011b). Besides IgG1 Met-431 oxidation, also fragmentation between Met-431 and His-432

upon MCO conditions was detected, which resulted in the formation of soluble and insoluble aggregates (Mozziconacci et al. 2016b).

Mechanisms of cysteine oxidation

Due to the nucleophilicity and the different oxidation states of the sulfur atom, Cys residues are the most oxidation-prone sites in proteins. Disulfides, which represent one of the most abundant oxidation products of Cys, may undergo type I photo-ionization, leading to the formation of radical ions ($RSSR^{\cdot+}$ and $RSSR^{\cdot-}$). Alternatively, they can also act as quenchers of excited states of Tyr and Trp residues. Radical disulfide anions ($RSSR^{\cdot-}$) may either dissociate into thiolates and thiyl radicals ($RS^{\cdot}$), or react with molecular oxygen to produce superoxide radical anions ($O_2^{\cdot-}$) (Scheme 3a) (Davies and Truscott 2001; Millington and Church 1997; Wardman and Vonsonntag 1995).

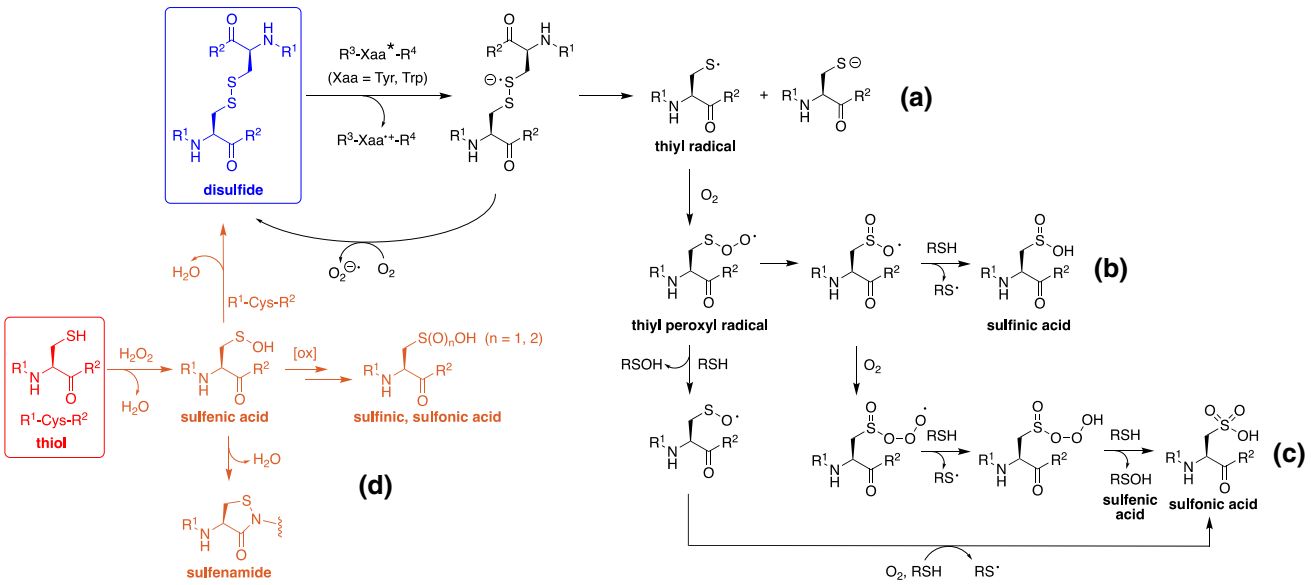
The thiyl radicals ($RS^{\cdot}$) can give rise to a series of reactions including hydrogen abstraction from C–H bonds or reactions with O_2 to generate an intermediate thiyl peroxyl radical ($RSOO^{\cdot}$) that rearranges into a sulphonyl radical ($RS(O)O^{\cdot}$) as precursor of sulfinic acid (RS(O)OH) (Scheme 3b). Alternatively, the formation of sulfenic (RSOH) and sulfonic ($RS(O)_2OH$) acids from $RSOO^{\cdot}$ has also been observed (Scheme 3c) (Becker et al. 1988; Millington and Church 1997; Schöneich 2012; Sevilla et al. 1988; Tamba et al. 1995; Wardman and Vonsonntag 1995).

The reaction of Cys with H_2O_2 in aqueous solution produces a sulfenic acid intermediate that, depending on the thiol density and pH of the microenvironment surrounding the Cys residue, may either form disulfide bonds or further oxidize to sulfinic and sulfonic acids, or undergo cyclization to sulfenamide (Scheme 3d).

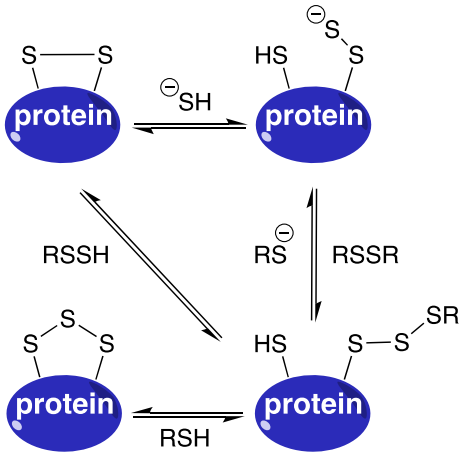
Noncanonical disulfide and trisulfide bridges in mAbs

Thiol–disulfide exchange reactions, which favor the formation of native from non-native disulfide bonds, occur during the oxidative folding of proteins and are usually mediated by specialized enzymes like protein disulfide isomerase (PDI) (Hudson et al. 2015; Moroder and Buchner 2008). In contrast, the accumulation of non-native disulfide bonds favors protein misfolding and aggregation (Hawe et al. 2009; Jordan et al. 1994). Occurrence of noncanonical disulfide bonds has been observed in mAbs and has been correlated with the existence of half molecules (Bloom et al. 1997; Schuurman et al. 2001), hybrids (Schuurman et al. 1999; Yoo et al. 2003), as well as structural isoforms presenting different hydrodynamic size, higher order structures, and potency (Dillon et al. 2008; Martinez et al. 2008).

Besides noncanonical disulfide bonds, also trisulfides have been detected in all subclasses of recombinant IgGs, which were mostly formed between light and heavy chains or two heavy chains to an extent directly dependent on the fermentation parameters employed (Gu et al. 2010). A non-enzymatic mechanism based on a thiol–disulfide exchange has been proposed (Nielsen et al. 2011), in which the reaction of a disulfide with a sulfhydryl anion (HS^-) generates a perthiolate and a thiol; the perthiolate can reduce another disulfide-containing molecule to form a mixed trisulfide that can further undergo reshuffling to a new trisulfide or a disulfide (Scheme 4).



Scheme 3 Possible oxidation processes of **a**–**c** cystine and **d** cysteine



Scheme 4 Possible thiol–disulfide exchanges leading to trisulfide bonds

Mechanisms of histidine oxidation

Oxidation of His occurs mainly via type II photo-oxidation or MCO mechanisms (Davies and Truscott 2001; Schöneich 2000). In the first case, the reaction follows a [2 + 4] cycloaddition mechanism, in which the imidazole ring of His reacts with $^1O_2$ and forms 2,4- or 2,5-endoperoxide intermediates, depending on the pH and, consequently, on the protonation state of the imidazole ring (Huvaere and Skibsted 2009; Liu et al. 2014a). At basic pH, the reaction pathway involves the formation of a 2,4-endoperoxide intermediate, which rapidly converts into 4-hydroxy-2-oxo-His (Scheme 5a). The latter presents an electrophilic site at C-5 that may undergo nucleophilic attack either by the α-amine to form the corresponding intra-residue bicyclic product, or by another nucleophile from the surrounding environment, giving rise to cross-linked products (Amano et al. 2014; Lei et al. 2017; Xu et al. 2017). Instead, water addition at C-5 (directly or via epoxide formation) leads to 4,5-dihydroxy-2-oxo-His that may further decompose to aspartic acid, asparagine and urea via the 4-hydroxy-2,5-dioxo-His intermediate (Scheme 5a) (Agon et al. 2006; Tomita et al. 1968).

Different products are obtained at neutral pH, where the 2,5-endoperoxide precursor converts into the corresponding 2-hydroxy-5-oxo-His and 5-hydroxy-2-oxo-His, which do not undergo any cyclization or other nucleophilic attack due to steric hindrance at the C-4 position (Scheme 5a).

In the case of MCO of His, the hydroxyl radical may react quite randomly and attack the imidazole ring of His at C-2, C-4 or C-5 positions to build 1,3-dihydro-2-oxo-His, and, in case of C-4 or C-5 addition, also other degradation products (Schöneich 2000) (Scheme 5b).

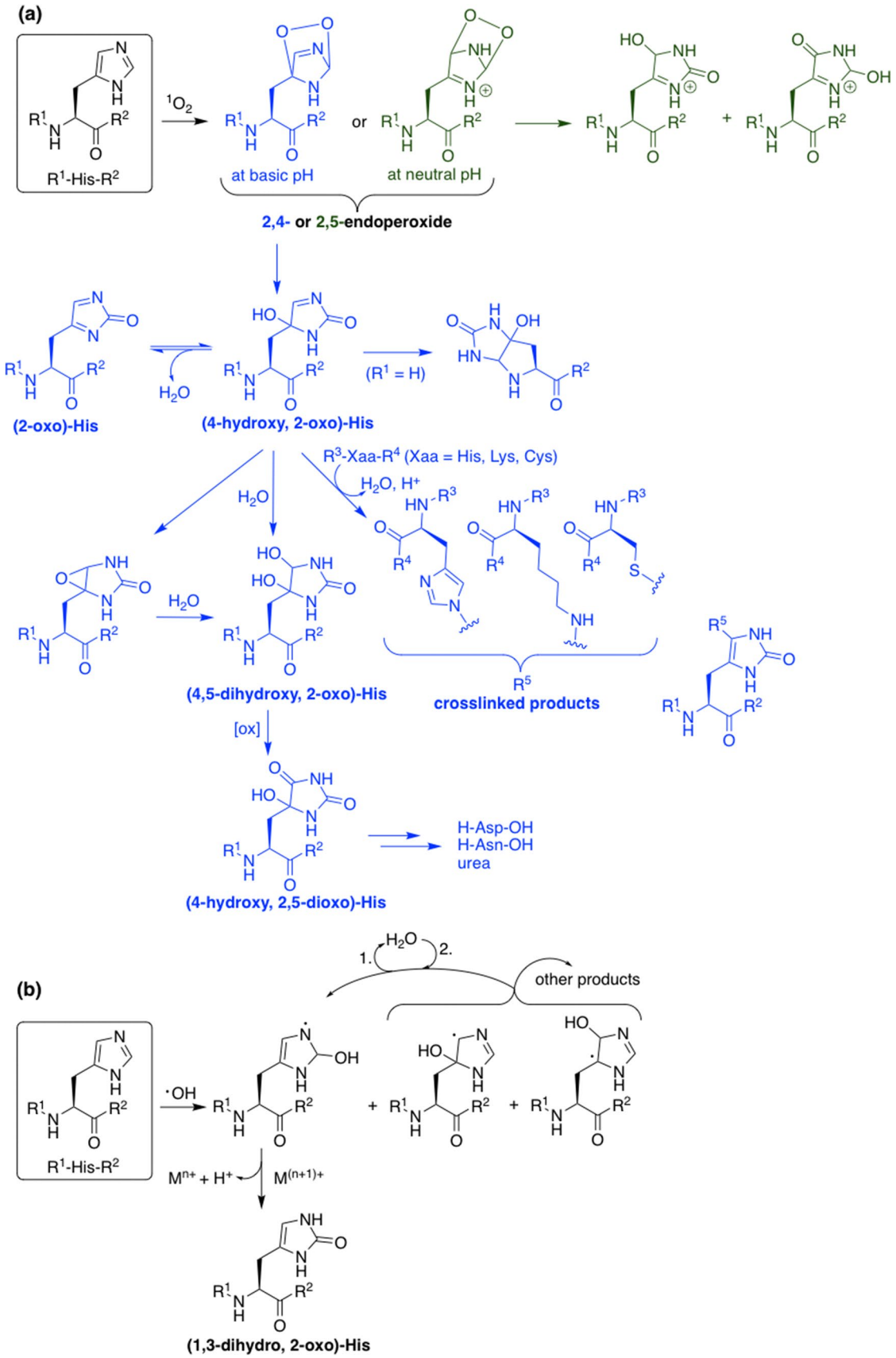
Susceptibility of biotherapeutics to histidine oxidation

His photo-oxidation was observed in an IgG1 mAb as a result of light exposure or of treatment with H_2O_2 in the presence of molybdate for $^1O_2$ generation, and it was shown to occur preferentially at His-289 due to the high solvent accessibility and, also, to the catalytic effect exerted by the surrounding amino acids (Amano et al. 2014). In another study, photocross-linking of the two IgG1 heavy chains via His-228 in the hinge region was detected, suggesting alternative processes for His oxidation to 4-hydroxy-2-oxo-His that then undergoes cross-linking (Liu et al. 2014b). Similarly, in high-molecular-weight (HMW) fractions of a 1-year-old IgG1 sample that was not light-stressed, His-228, His-289, and His-437 were identified as hot-spots for cross-linking reaction with Lys or Cys (Xu et al. 2017).

For a monoclonal IgG2, 1,3-dihydro-2-oxo-His was the preferential MCO product of His-304 and His-428 upon treatment with Cu^{2+}/ascorbate, which was attributed to a putative copper-binding site between these His residues and the neighboring Met-246 (Luo et al. 2011). In the case of the human growth hormone, treatment with Cu^{2+}/ascorbate resulted in extensive oxidation of His-18 and His-21, which are both located in the metal-binding site. While His-21 provided also oxidation products other than 1,3-dihydro-2-oxo-His, thus suggesting a C-4 and/or C-5 addition, His-18 was quantitatively oxidized to 1,3-dihydro-2-oxo-His, which is indicative of a direct C-2 addition and/or of initial addition on C-4 and/or C-5 followed by a hydroxyl radical shift mediated by efficient water elimination/addition (Schöneich 2000; Zhao et al. 1997) (Scheme 5b). MCO products of His were also observed in insulin samples, where the reaction selectively occurred across the B chain with formation of 1,3-dihydro-2-oxo-His at both residues involved in zinc ion binding (Hovorka et al. 2002; Sadineni and Schöneich 2007).

Mechanisms of tryptophan oxidation

With its indole group, Trp represents the strongest chromophore in proteins, and its photo-oxidation gives rise to a complex mixture of products (Davies 2003, 2004; Ehrenshaft et al. 2015; Langlots et al. 1986; Pattison et al. 2012; Saito et al. 1977). Type II photo-oxidation of Trp follows a [2 + 2] cycloaddition mechanism involving a C-2, C-3-dioxiethane intermediate that converts into *N*-formylkynurenine (NFK), kynurenine (Kyn) by deformylation of NFK, or a diastereomeric mixture of oxindolylalanine (Oia) and dioxindolylalanine (diOia) (Zhang et al. 1993). Alternatively, $^1O_2$ may add to the indole group through an ene-reaction giving tryptophanyl-hydroperoxide, followed by a nucleophilic attack of the hydroperoxide or of the α-amino group at the imine, which leads, respectively, to



Scheme 5 Possible oxidation processes of His by **a** photo-oxidation and **b** MCO

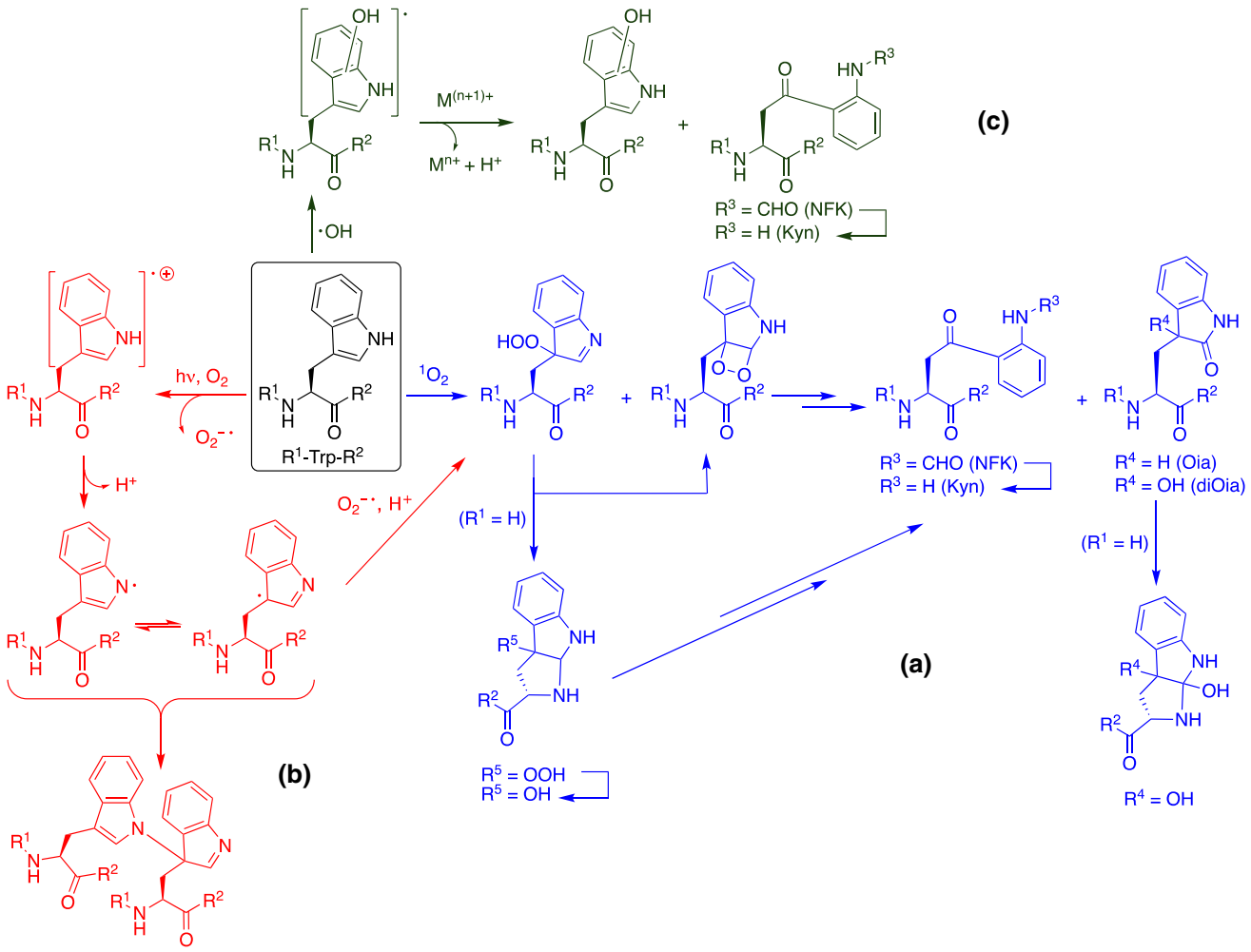
the dioxiethane intermediate or to a pyrroloindole derivative (Scheme 6a) (Gracanin et al. 2009; Nakagawa et al. 1977).

Furthermore, type I photo-oxidation of Trp results in a radical cation ($Trp^{\cdot+}$) that undergoes either Cα-Cβ homolytic cleavage (Schöneich 2018) or deprotonation of the NH group of the indole ring to generate a tryptophanyl (indolyl) radical ($Trp^{\cdot}$) (Creed 1984a). This species may capture a superoxide radical anion ($O_2^{-\cdot}$) to form tryptophanyl-hydroperoxide that may undergo intramolecular cyclization to generate a C-2, C-3-dioxetane intermediate or a pyrroloindole derivative (Aspee and Lissi 2000). Alternatively, also dimers and trimers arising from the tryptophanyl radical ($Trp^{\cdot}$) have been detected upon photo-oxidation in the presence of riboflavin (Silva et al. 2019) (Scheme 6b).

MCO mechanisms have been reported, as well, which lead to oxidized Trp species including hydroxy-Trp, NFK, and Kyn (Finley et al. 1998).

Susceptibility of mAbs to tryptophan oxidation

Oxidative modification of Trp has been shown to depend both on the identity of the neighboring amino acids and on its location in the protein structure (Pigault and Gerard 1984; Tassin and Borkman 1980), and ad-hoc developed RP-HPLC-based methods have been employed for its determination and characterization in biopharmaceuticals (Yang et al. 2007). Irradiation of a mAb with near UV–visible light resulted in extensive oxidation of Trp-50 and Trp-104 in the heavy chain, and of Trp-90 in the light chain, with Trp-104 exhibiting the fastest oxidation rate, with concomitant formation of Kyn, NFK, 5-OH-Trp, Oia, and diOia (Li et al. 2014). Similarly, exposure of the humanized mAb MEDI-493 to UV light caused substantial oxidation of heavy-chain Trp-105, which was accompanied by a substantial decrease in binding affinity for the corresponding antigen (respiratory syncytial virus F protein), with a significant decrease in potency (Wei et al. 2007). Also in the case of



Scheme 6 Possible oxidation processes of Trp by **a**, **b** photo-oxidation and **c** MCO

the recombinant IgG1 antibody HER2, progressive oxidation of the solvent-exposed Trp-32 induced by treatment with *t*BHP was accompanied by a decrease in the binding affinity to the own target. Interestingly, the complementary determining region (CDR), where Trp-32 is located, was found to be more susceptible to the oxidation conditions used than the heavy-chain Met-429 and Met-107 (Hensel et al. 2011).

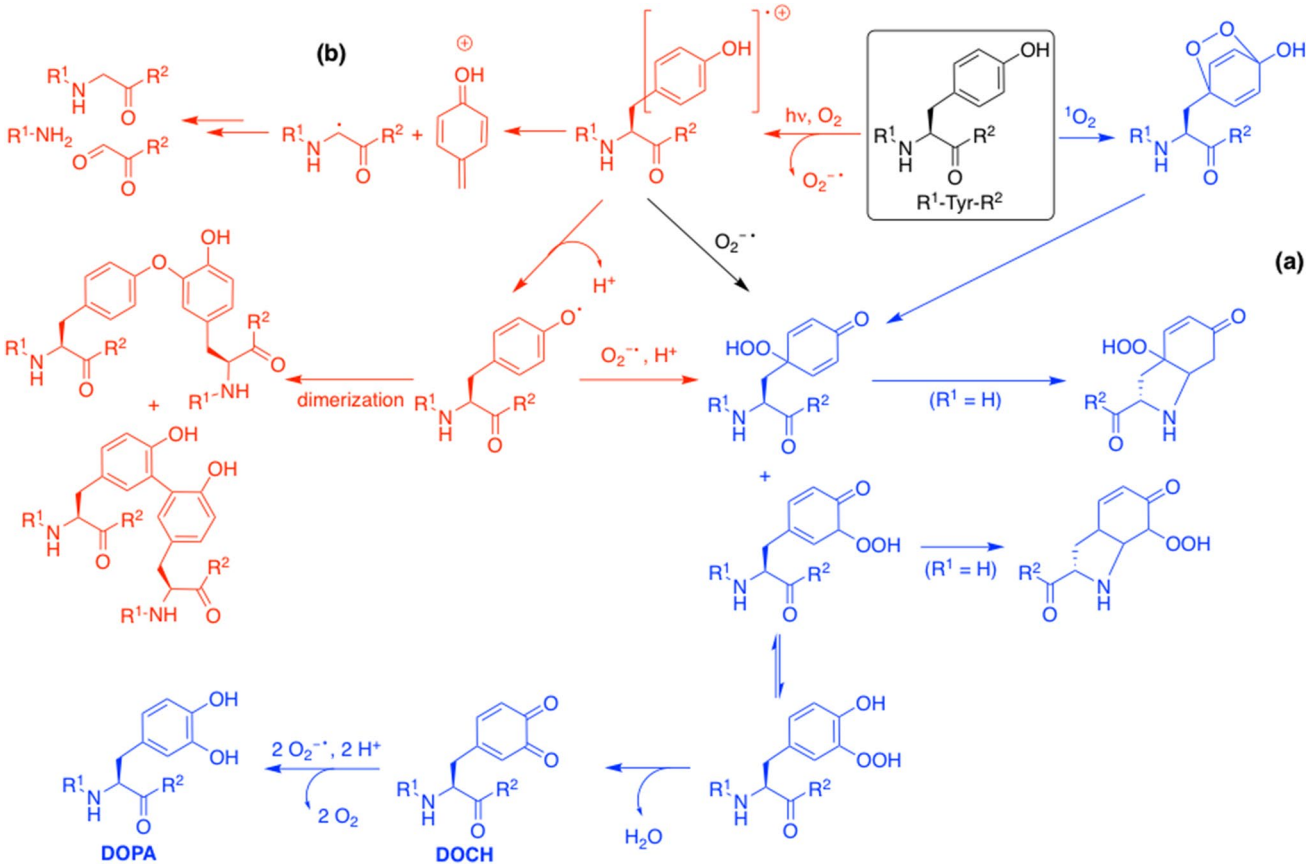
Mechanisms of tyrosine oxidation

Analogously to Trp, photo-oxidation of Tyr is one of the major oxidative degradation processes in peptides and proteins. $^1O_2$ mediated oxidation was reported to proceed under basic conditions with the formation of unstable endoperoxides that rearrange into hydroperoxides. The latter contain a Michael acceptor system that can react with various nucleophiles leading to intra-residue cyclization or inter-residue cross-linking (Scheme 7a) (Rizzuto and Spikes 1977; Wright et al. 2002).

Oxidation of Tyr side chains may also be mediated by radical species, resulting either from MCO (Ali et al. 2004) or type I photo-oxidative events (Creed 1984b). In the latter case, the reaction mechanism proceeds analogously to that observed for Trp, with the formation of a Tyr radical cation. This may undergo the homolytic cleavage of the Cα–Cβ bond, resulting in a protonated quinone methide and a Cα-glycyl radical (Kang et al. 2019), or it may build a phenoxyl radical through proton loss (Scheme 7b). The phenoxyl radical may either dimerize to give C–O or C–C dityrosine adducts (Aeschbach et al. 1976; Garrison 1987), or further oxidize to form *p*- or *o*-hydroperoxide intermediates that may react with vicinal nucleophilic residues (Garrison 1987; Ito et al. 1988; Moller et al. 2012; Winterbourn et al. 2004). Alternatively, the *o*-hydroperoxide may form an *o*-benzoquinone (DOCH) that is susceptible to Michael additions (Ito et al. 1984), or it may further convert into dihydroxy phenylalanine (DOPA) (Bielski et al. 1985; d'Alessandro et al. 2000; Song and Buettner 2010).

Susceptibility of biotherapeutics to tyrosine oxidation

Tyr oxidation has been detected in protein therapeutics after oxidative treatments. For example, in interferon β, oxidative modification of Tyr was induced with Cu^{2+}/ascorbate and resulted in the formation of highly immunogenic aggregates



Scheme 7 Possible type II (**a**) and I (**b**) photo-oxidation processes of Tyr

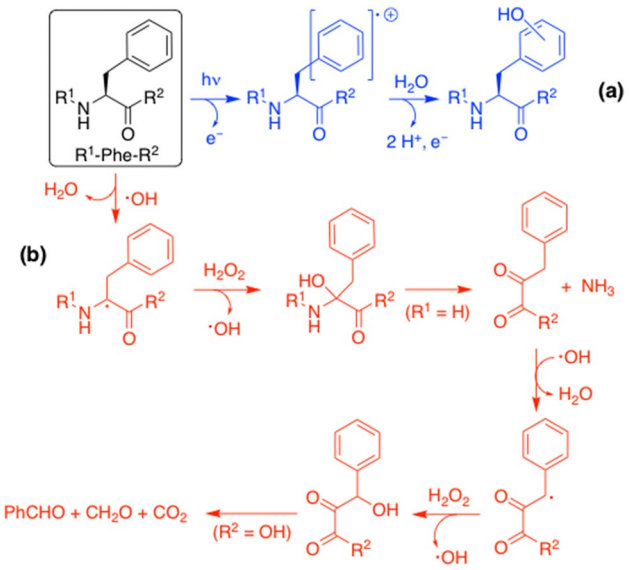
arising from 1,4- or 1,6-type addition of the N-terminus or Lys-105 side chain to the *o*-benzoquinone degradation product of Tyr-20, Tyr-60, Try-126, or Phe-11 (Torosantucci et al. 2013).

Oxidation of insulin with Cu^{2+}/ascorbate resulted in the formation of the *o*-benzoquinone DOCH and DOPA at Tyr positions B16, B26, A14, and A19, with subsequent formation of cross-linked aggregates (Torosantucci et al. 2012). In another example, MCO of insulin with H_2O_2/Cu formed dityrosine, which resulted in a significant decrease in glucose oxidation capability (Olivares-Corichi et al. 2005), similarly to what observed by comparing the decrease in biological activity of insulin following either Fenton oxidation or incubation in diabetic patient´s plasma (Montes-Cortes et al. 2010). UV light treatment of the Fc region of IgG4 was shown to produce degradation products of Tyr at positions 300, 373, and 436, corresponding to glycine and glyoxal amide (Kang et al. 2019).

Mechanisms of phenylalanine oxidation

Oxidation of Phe follows predominantly photo-oxidative mechanisms (Scheme 8) (Bent and Hayon 1975). Absorption of UV light may induce photodissociation generating the benzyl radical, or photo-ionization yielding, like in the case of Trp and Tyr, a radical cation and its hydroxylated ring products that, then, undergo degradation pathways in analogy to those observed for Tyr (Davies and Gilbert 1992).

MCO of Phe has also been observed (Ashraf et al. 1980): in this case, direct reaction with the hydroxyl radical was suggested to occur at the Cα position with formation of an α-keto acid that degrades to aldehyde products and CO_2.



Scheme 8 Possible type I (**a**) and MCO (**b**) processes of Phe

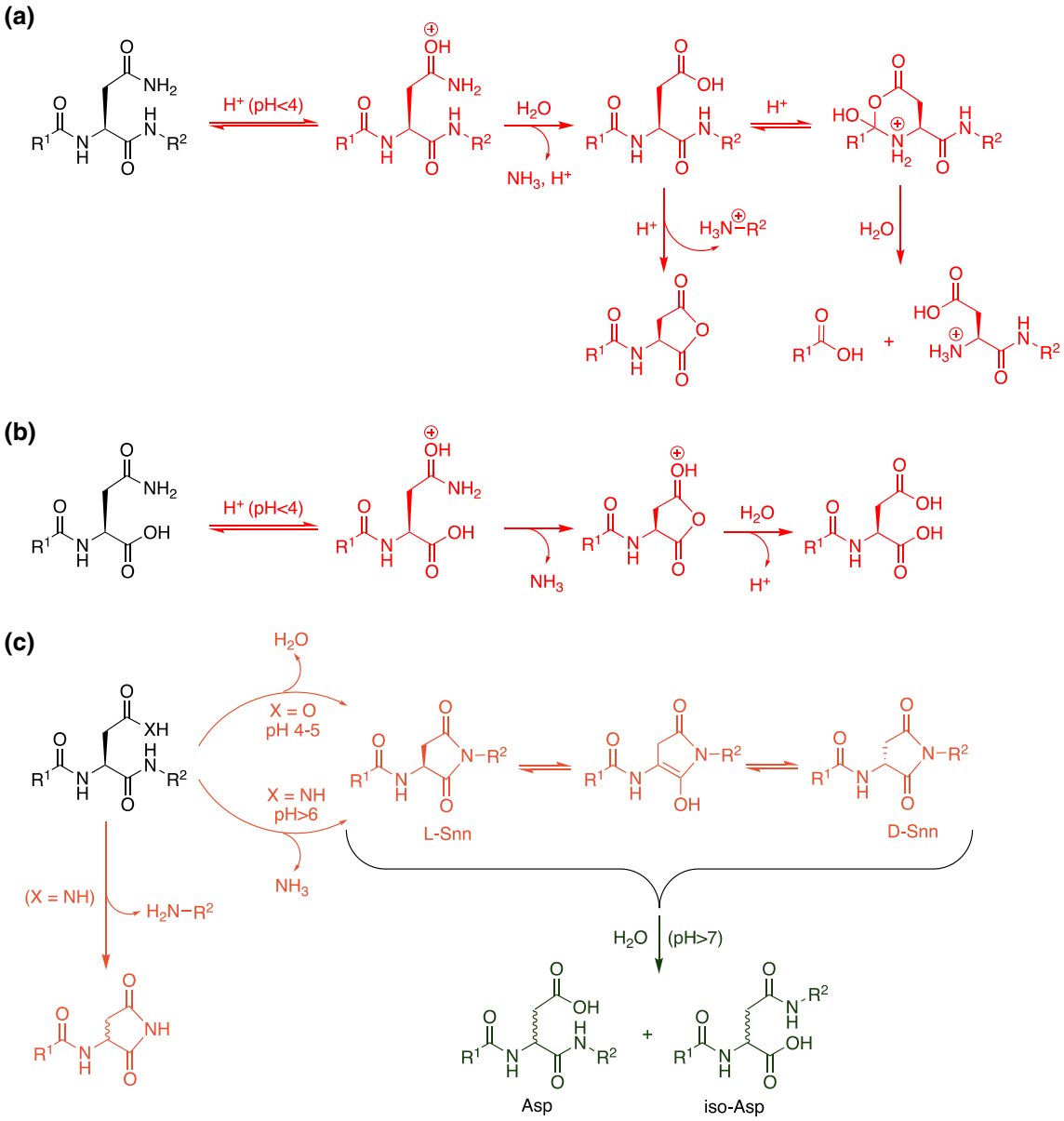
Intra- and inter-residue cyclizations upon loss of water or ammonia

Mechanisms of asparagine and aspartic acid degradation

Both Asp and Asn residues are subjected to spontaneous degradation reactions that may lead to a variety of side products, depending on the environmental conditions and the local conformation (Clarke 1987; Geiger and Clarke 1987; Patel and Borchardt 1990b). Under highly acidic conditions (pH < 4), Asn residues may be converted into Asp residues by acid-catalyzed deamidation (Oliyai and Borchardt 1993; Patel and Borchardt 1990a), which further promotes the acid-catalyzed formation of succinic anhydride upon cleavage of the peptide bond following Asp, especially when the n + 1 position is occupied by Pro (Marcus 1985) (Scheme 9a). Peptide-bond cleavage may occur under acid conditions also before Asp, when Gly is present at the n-1 position, probably by formation of a six-membered cyclic intermediate (Marcus 1985; Patel and Borchardt 1990b) (Scheme 9a).

C-terminal Asn residues may be deamidated under acidic conditions also by a mechanism involving the formation of a succinic anhydride intermediate via intramolecular nucleophilic catalysis (Scheme 9b) (Darrington and Anderson 1994).

Under mildly acidic conditions (pH 4–5), Asn residues are mainly stable, whereas Asp residues are prone to form the succinimide (Snn) intermediate (Oliyai and Borchardt 1993; Patel and Borchardt 1990a). At pH > 6, the formation of the Snn intermediate becomes significant also for Asn (Patel and Borchardt 1990b). According to a generally recognized mechanism, the reaction proceeds through a nucleophilic attack of the peptide bond on the β-carbonyl side chain of Asn or Asp to form a metastable tetrahedral intermediate, from which ammonia or water is eliminated (Aylin et al. 2003; Capasso et al. 1989, 1991b, 1993; Oliyai and Borchardt 1993; Patel and Borchardt 1990a) (Scheme 9c). Interestingly, deamidation of Asn residues via the Snn intermediate is kinetically favored around pH 6 or higher (Patel and Borchardt 1990b), whereas it becomes less favored for Asp, which reflects the negative effect of the increased ionization of the aspartyl side chain (Capasso et al. 1992; Oliyai and Borchardt 1993; Patel and Borchardt 1990a). Once formed, the Snn intermediate undergoes water attack on either side of the imide nitrogen, Cα or Cβ, followed by breakage of the corresponding C–N bond to lead to a mixture of Asp and iso-Asp, the latter being produced in a two-to-fourfold excess (Xie et al. 1996). Furthermore, due to favorable inductive factors and resonance stabilization effects of the adjacent nitrogen



Scheme 9 Mechanisms of deamidation **a**–**c** of Asn and dehydration **c** of Asp

and β-carbonyl carbon, respectively, the Snn structure has inherent tendency to undergo base-induced racemization at Cα carbon (Radkiewicz et al. 1996).

Interestingly, Snn or succinic anhydride formation by intra-residue cyclization of Asn or Asp followed by peptide-bond cleavage was also observed, especially when the competitive mechanism leading to Asp and iso-Asp formation was slowed down by the presence of bulky residues at the n + 1 position, such as Thr, Val, Leu, or Pro (Geiger and Clarke 1987; Patel and Borchardt 1990b; Tyler-Cross and Schirch 1991), as well as of His that, besides the steric hindrance, also acts as general base catalyst at neutral or basic pH (Goolcharran et al. 2000) (Scheme 9c).

Beside the pH, other variables related to both external conditions and intrinsic properties of the chemical environment surrounding the Snn-prone site may affect the course of the reaction and the product profile. To this regard, the steric effect and electron-withdrawing properties of the amino acid adjacent to the sensitive site play a major role. Accordingly, amino acids with poor steric hindrance, above all Gly, at the n + 1 position, give the highest extent of Snn formation, whereas more sterically hindered residues like Leu, Pro, Ile, Phe, and Val offer higher degree of protection (Behrendt et al. 2016; Patel and Borchardt 1990b; Robinson et al. 1973). Residues presenting a cationic side chain at physiological pH such as Lys and Arg may promote Snn formation

by stabilization of the anionic tetrahedral intermediate and/or by general acid catalysis. In the case of His, both general acid and base catalysis can be observed, depending on the pH (Goolcharran et al. 2000). Other neighboring groups, including the β-hydroxyl groups of Ser or Thr at the n + 1 position (Patel and Borchardt 1990b; Robinson et al. 1973), have showed catalytic effects in the formation of Snn.

Susceptibility of mAbs to asparagine and aspartic acid degradation

Asn and/or Asp degradation products have been observed in proteins, including mAbs, where they have been found both in Fab and Fc domains, and their impact on mAb stability, potency, and/or safety has been considered as the object of study (Reissner and Aswad 2003; Wang 1999). In such complex systems, structural factors as well as the extent of solvent exposure may greatly affect the degradation of Asn and Asp residues (Wakankar et al. 2007). For example, the CDR region of mAbs contains several Asp and Asn residues that may be considered hot-spot degradation points, depending on their location within the protein (Huang et al. 2005; Vlasak et al. 2009). In particular, Asn-30, Asn-55, and Asp-102 were shown to be particularly susceptible to iso-Asp conversion, with consequent decrease in target binding affinity and potency of an antibody against HER/neu (Harris et al. 2001).

Deamidation has also been detected in the Fc region, particularly in the sequence motif Leu–Asn–Gly–Lys in the CH2 domain (Chelius et al. 2005; Mukherjee et al. 2010) and at Asn-384 and Asn-389 in the CH3 domain. Interestingly, the latter two residues converted mainly into iso-Asp and Asp, respectively, which was attributed to a specific effect of the local structure on the Snn hydrolysis pathway (Sinha et al. 2009). Within the CH3 domain, deamidation events were also reported along the C-terminal part, particularly at Asn-434 (Khawli et al. 2010), which is of biological relevance, as it has been shown that this residue is included in the binding site of human IgG1 to the FcRn (Shields et al. 2001; Ying et al. 2014). Therefore, alterations of Asn-434 may eventually alter the antibody presence in the circulation. However, it should be also mentioned that charge variants of a recombinant humanized IgG1 mAb did not show significantly different pharmacokinetic properties in serum (Khawli et al. 2010).

Mechanisms of diketopiperazine formation

Diketopiperazine (DKP) formation has been found in tryptic digests (Jornvall 1974), and also as degradation product of peptide solutions or formulations upon long storage (Oyler et al. 1991; Sepetov et al. 1991; Straub et al. 1995). The intramolecular cyclization proceeds by initial *trans* → *cis* isomerization of the peptide bond between the first two residues, followed by nucleophilic attack of the α-amine of the first residue on the α-carbonyl of the second residue to produce a zwitterionic intermediate that evolves into the DKP upon elimination of the third residue (Capasso et al. 1998) (Scheme 10). Interestingly, the zwitterionic intermediate has been proposed to rearrange at high temperatures (100 °C or higher) and pH > 6 to possible bicyclic intermediates that convert into the inverted linear dipeptidyl moiety or to the linear dipeptidyl moiety with epimerization of the first residue (Sepetov et al. 1991; Steinberg and Bada 1981, 1983) (Scheme 10a).

DKP formation can be both general base- and general acid-catalyzed (Capasso et al. 1998; Goolcharran and Borchardt 1998; Suzuki et al. 1981), although at basic pH values the *trans* → *cis* isomerization of the peptide bond becomes the rate-limiting step (Capasso et al. 1998). Moreover, the cyclization is favored by the presence of Pro or Gly at the second position (Gisin and Merrifield 1972).

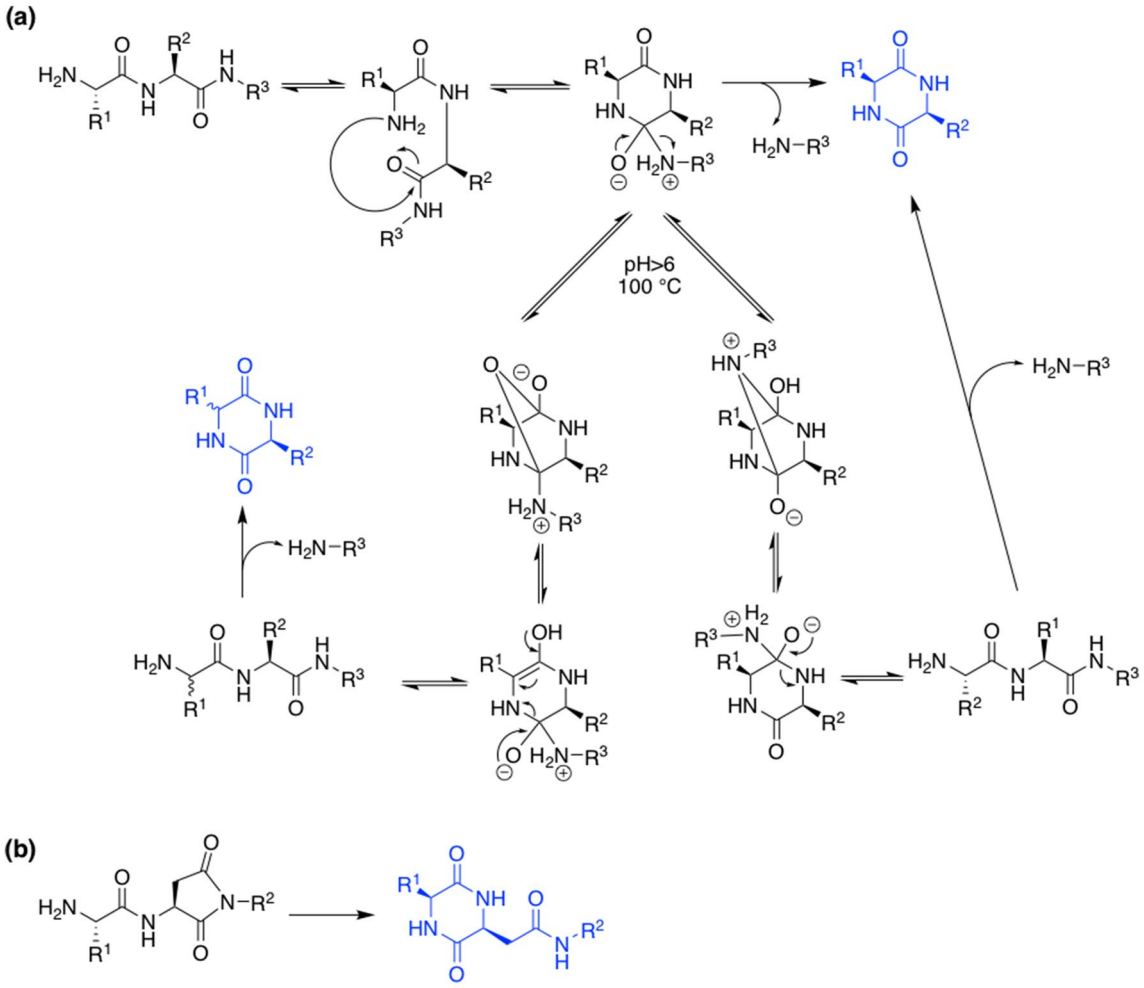
Interestingly, DKP formation has also been observed in peptides containing amino-acid residues prone to Snn formation at position 2, as a result of the intramolecular attack of the free N-terminal amine on the α-carbonyl of the Snn ring (Scheme 10b) (Brückner et al. 2012; Dehart and Anderson 2007; Jornvall 1974; Schon and Kisfaludy 1979).

Mechanisms of glutamine and glutamic acid degradation

As already reported for Asn residues, also Gln residues undergo direct deamidation under acidic conditions in a sequence-independent manner (Joshi et al. 2005; Robinson and Rudd 1974) (Scheme 11a). At pH close to 6, Gln side chains exhibit the highest stability (Scotchler and Robinson 1974). At neutral and basic pH, Gln deamidation proceeds via formation of a six-membered glutarimide intermediate (Scotchler and Robinson 1974; Robinson and Rudd 1974; Robinson et al. 1973). Water attack on one of the two carbonyl groups affords the corresponding γ-Glu-peptide or α-Glu-peptide, with a preference for the γ-residue (Scheme 11b) (Capasso et al. 1991a).

However, the formation of glutarimide is much less favored than the formation of Snn due to the higher distance of the γ-carbonyl group from the backbone (Robinson and Rudd 1974; Robinson et al. 2004), in accordance with the fact that Gln deamidation has been mainly detected in long-lived proteins like βB2-crystallin in the human lens, which causes dimer destabilization (Lampi et al. 2006).

Contrarily to the deamidation of internal Gln residues, the deamidation of N-terminal Gln residues may easily occur via intra-residue cyclization, which results in the formation of pyroglutamate (pGlu). Also N-terminal Glu residues may convert to pGlu, especially at pH around 4



Scheme 10 Mechanisms of DKP formation **a** at positions 1 and 2 of a peptide chain, and **b** in the presence of Snn at position 2

or 8, whereas the conversion slows down around pH 6–7, suggesting weak-acid/base catalysis (Chelius et al. 2006; Dimarchi et al. 1982; Yu et al. 2006) (Scheme 11c).

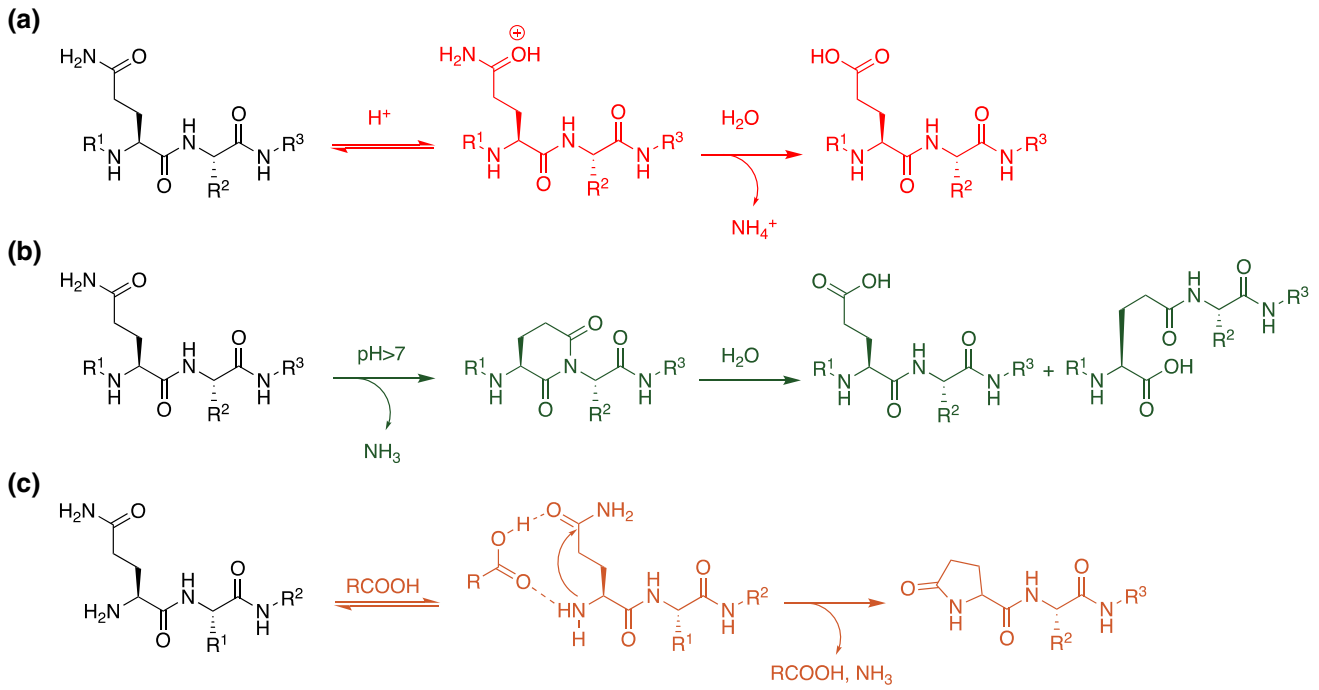
Due to the more favored formation of the five-membered pyrrolidone than that of the six-membered glutarimide, degradation of Gln and Glu residues is mainly restricted to N-terminal Gln/Glu (Kumar and Bachhawat 2012; Perez-Garmendia and Gevorkian 2013). However, it should be taken into account that pGlu formation is not only a spontaneous reaction, but it can also be catalyzed by glutaminyl cyclase, an enzyme that is present in plants and mammalians (Fischer and Spiess 1987; Schilling et al. 2004). Indeed, in the case of the amyloid β (Aβ) peptide, it was shown that inhibition of glutaminyl cyclase in vivo decreased the amount of the pGlu-containing Aβ (Schilling et al. 2008), which is characterized by lower solubility (Schlenzig et al. 2009), higher proteolytic resistance (Rink et al. 2010) and more toxicity (Russo et al. 2002).

Susceptibility of mAbs to pGlu formation

The presence of pGlu is frequently detected in recombinant mAbs, which is mainly attributed to spontaneous cyclization during both production (fermentation and purification) and storage (Chelius et al. 2006; Dick et al. 2006; Yu et al. 2006), and it is a reason for charge heterogeneity (Lyubarskaya et al. 2006; Moorhouse et al. 1997; Rehder et al. 2006). Nevertheless, no significant differences in comparison to the Gln/Glu forms have been reported with regard to potency in vitro (Lyubarskaya et al. 2006), or clearance in humans (Liu et al. 2011).

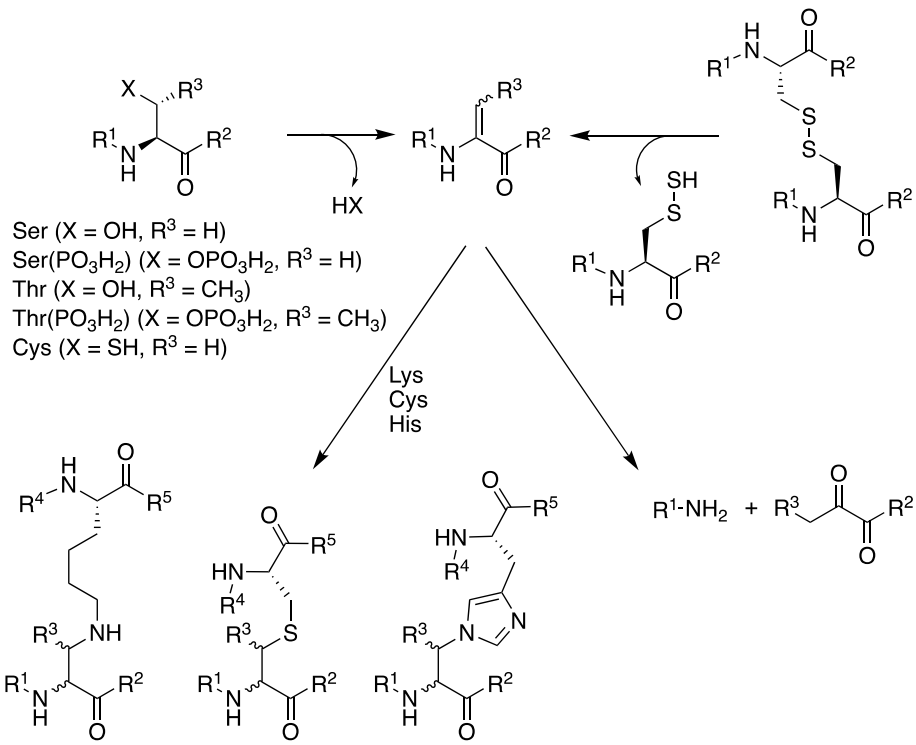
β-Elimination reactions of β-hydroxyl- and β-sulfhydryl-amino acids

Ser, Thr, Cys, and cystine may undergo an acid- or base-catalyzed β-elimination reaction that results in dehydroalanine (ΔAla), β-methyl-ΔAla, and, in case of cystine,



Scheme 11 Mechanisms of **a**, **b** deamidation of Gln, and cyclization of N-terminal Gln

Scheme 12 β-Elimination of Ser, Thr, Cys, and cystine



also of thiocysteine (Scheme 12) (Bar-Or et al. 2008; D'Hondt et al. 2014a). Due to the sp^2 hybridization of the α-carbon, the α,β-dehydro amino-acid residue has a planar geometry, which forces co-planarity of the two planar peptide units centered at the sp^2 α-carbon. This induces backbone conformations that are unusual for the native backbone (Ajo et al. 1980), which may influence the structural features of peptides and proteins (Palmer et al. 1992). Furthermore, due to the presence of the α,β-unsaturated carbonyl moiety, α,β-dehydro amino-acid

residues enhance the reactivity of peptides and proteins: indeed, they may form covalent cross-linking via Michael addition of suitable nucleophiles like those of Lys, Cys, or His side chains, leading to lysinoalanine, lanthionine, and histidinoalanine linkages (Cohen et al. 2007; Costantino et al. 1994; Linetsky et al. 2004; Nashef et al. 1977), respectively. In addition, the by-product thiocysteine may trigger disulfide bonds reshuffling in cystine-containing peptides and proteins (Scheme 4) (Costantino et al. 1994).

Furthermore, ΔAla derivatives may lead to backbone cleavage between the αN and αC of the ΔAla residue, resulting in C-terminally amidated and N-terminally pyruvoylated fragments (Cohen et al. 2007; D'Hondt et al. 2014b; Patchornik and Sokolovsky 1964; Sokolovsky et al. 1964; Wisniewski et al. 2013).

Conclusions

Spontaneous modifications of amino acids are a well-known problem in peptide and protein research. However, it is obvious that they become very important, when peptides and proteins are used as drugs. In this case, the presence of spontaneous chemical changes and their biological effects must be carefully investigated (D'Hondt et al. 2014a; Wu et al. 2017). As a matter of fact, a lot of efforts are made to develop analytical tools and chemical protocols for the detection of spontaneous modifications in proteins and biopharmaceuticals (Alcock et al. 2018; Beck et al. 2013; Berkowitz et al. 2012; Forstenlehner et al. 2015; Grassi et al. 2017; Kettenhofen and Wood 2010; Regl et al. 2017; Schweida et al. 2019). Moreover, (semi)synthetic approaches are applied to reproduce proteins containing the spontaneous modification of interest at selected positions, to evaluate its impact on the protein structure and biology: for example, an explorative study has been conducted to assess the synthetic accessibility of the Fc CH3 IgG1 region containing a modified residue, i.e., Met(O), at the desired position, to avoid the post-production treatment with strong oxidants (Grassi et al. 2018). Furthermore, the semisynthetic approach has been used to prepare the two site-selective glycated proteins Hsp27 (Matveenko et al. 2016) and Tau (Ellmer et al. 2019): glycation refers to a class of non-enzymatic modifications of biomolecules containing nitrogen nucleophiles that react with the carbonyl group of ketoses or aldoses to build a Schiff base. The latter can slowly isomerize to the Amadori product (from aldoses) or Heyns–Carson product (from ketoses), which undergo further transformations to so-called advanced glycation end-products (AGEs), like argpyrimidine and Nε-(carboxymethyl)-lysine. AGEs are believed to play a major role in aging and pathophysiological processes (Fournet et al. 2018). Hsp27 (heat-shock protein 27) is a chaperone protein that plays a role in the regulation of apoptosis in cancer cells, e.g., by inhibiting cytochrome *c*-mediated caspase activation and promoting cell survival. However, a glycated form of Hsp27 containing argpyrimidine at position 188 has been also isolated, which, contrarily to unmodified Hsp27, forms significantly smaller oligomers and is not able to counteract caspase activation by cytochrome *c* (Sakamoto et al. 2002). Accordingly, the semisynthetic glycated variant has shown reduced chaperone activity in vitro (Matveenko et al. 2016). Tau is a protein that regulates microtubule assembly and disassembly in neurons and undergoes a number of PTMs, including phosphorylation and glycation, which may have an effect on its propensity to form pathogenic fibrils (Liu et al. 2016). A semisynthetic variant of Tau4 containing Nε-(carboxymethyl)-lysine at position 294 has shown to negatively affect tubulin polymerization while displaying very similar fibrillization to the non-glycated variant, a behavior that has resulted to be opposite to that of phosphorylated Tau4 variants (Ellmer et al. 2019).

While many more spontaneous reactions of amino-acid residues are known than those mentioned, like reversible hydrogen-transfer reactions involving thiyl radicals in Cys-containing peptides and proteins (Mozziconacci et al. 2010, 2011; Steinmann et al. 2017), still many others are expected to occur, which will need to be detected and explored both mechanistically and biologically (Schöneich 2017). Future research in this field will enormously support the development of safe and potent peptide and protein drugs.

Acknowledgements Open access funding provided by Paris Lodron University of Salzburg. C. C. kindly acknowledges the Land Salzburg and the Paris Lodron University of Salzburg for financial support. The financial support by the Austrian Federal Ministry of Science, Research, and Economy and by a Startup Grant of the State of Salzburg is gratefully acknowledged.

Compliance with ethical standards

Conflict of interest The salary of L. G. was fully funded by the Christian Doppler Laboratory for Biosimilar Characterization, which is partly supported by Novartis and Thermo Fisher Scientific. The authors declare no other competing financial interest.

Research involving human participants This is a review paper without participation of humans.

Informed consent No inform consent was required (see above).

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