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Mechanisms of metal-catalyzed oxidation of histidine to 2-oxo-histidine in peptides and proteins☆

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Abstract

The metal-catalyzed oxidation of histidine (His) to 2-oxo-histidine (2-oxo-His) represents an important pathway of protein oxidation in vivo and in vitro. In the pharmaceutical literature this pathway has received less attention. However, this fact may not necessarily represent reality as, in some cases, the analysis of His oxidation in proteins may be compromised by aggregation and precipitation of the target protein. For predicting the susceptibility of His residues in proteins it is important to understand in detail how protein sequence and conformation control the mechanisms of His oxidation to 2-oxo-His, reviewed in this paper. © 2000 Elsevier Science B.V. All rights reserved.

Keywords: Histidine; Metal-catalyzed oxidation; 2-Oxo-histidine; Human growth hormone; Hydroxyl radical

1. Introduction

Protein oxidation represents a major pathway of protein degradation in pharmaceutical formulations [1,2]. However, unlike hydrolytic (solvolytic) mechanisms such as deamidation, β-elimination, or diketopiperazine formation, the underlying mechanisms are known in much less detail. This is, in part, due to the fact that for most pharmaceutical formulations, in the absence of any applied exogenous stress, the participating oxidizing species are not well defined. Furthermore, oxidation reactions are significantly affected by protein sequence and structure. Additional complexities are introduced when oxidation reactions are catalyzed by transition metals. Such reactions may predominantly affect amino acid residues directly involved in metal-binding or located at or in close vicinity of the metal-binding site [3,4]. Thus, during metal-catalyzed oxidation (MCO) the most oxidation-sensitive residues do not necessarily have to be present on the surface of the protein. The majority of research on the MCO of proteins has been published in the biochemical literature as MCO of proteins is an important phenomenon of oxidative stress and biological aging [5,6]. Here, histidine (His) has been identified as one amino acid residue particularly susceptible to MCO in vivo,

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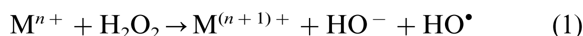
e.g. for Cu,Zn superoxide dismutase (Cu,Zn SOD) [7], and in vitro, e.g. for glutamine synthetase [8], Cu,Zn SOD [9], Fe^{2+} dehydrogenases [10], glycated insulin [11], and low density lipoprotein (LDL) [12]. In fact, conversion of His especially to 2-oxo-His (structure 1 in Scheme 1) has been suggested as an important marker for oxidative stress in vivo [13]. In the pharmaceutical literature, the potential modification of His, possibly to 2-oxo-His, has received little attention though recently 2-oxo-His formation has been demonstrated for the metal-catalyzed photodegradation of recombinant human growth hormone (hGH) [14]. However, the lack of examples for His modification in pharmaceutical formulations may not necessarily reflect reality; it is possible that, in some cases, the detection of His modification may require experimental methods which are not necessarily considered standard techniques in quality control (see below). For example, during our mechanistic analysis of His oxidation in hGH [15] all reaction products were easily analyzed by standard proteolytic techniques combined with NMR and mass spectrometry. However, for human relaxin the MCO of His resulted in a pH-dependent non-covalent aggregation and/or precipitation, and the protein precipitates had to be resolubilized for product analysis [16]. Amino acid analysis indicated that the MCO of recombinant human brain-derived neurotrophic factor (BDNF) led to a significant modification of His. However, though proteolytic mapping with Endo-Lys-C confirmed the loss of His-containing peptide fragments it failed to reveal any product peptide sequences [17].

Our goal is a detailed characterization of (1) the chemical mechanisms involved in the MCO of His; (2) the effects of protein sequence and structure on product formation from His oxidation, and (3) the biophysical consequences of His oxidation for individual proteins (for example, whether His oxidation affects the solubility of the protein). With such information, based on the sequence and conformation of a protein, His oxidation and its consequences may be predicted to a certain extent which would be of great assistance for the design of formulation conditions and analytical methodology for quality control. This article focuses on the chemical mechanisms of MCO of His to 2-oxo-His in proteins, including hGH, and the effect of conformation on product formation.

2. Metal-catalyzed oxidation of His

It is almost impossible to prevent the contamination of protein formulations with trace impurities of transition metals as most of the individual components of a formulation, e.g. buffer, excipients, protein, have quite significant affinity to metals. Only traces of transition metals are necessary for a rather efficient MCO.

When glutamine synthetase was exposed to MCO, the chemical modification of one mol His/mol protein (out of 16 mol His/mol protein) caused the inactivation of the enzyme, whereas other potentially oxidation-sensitive residues such as Tyr, Trp, Cys and Met remained unchanged [8]. Essentially two individual His residues were modified, one of which was characterized as His_{269}, located in the sequence Met-His_{269}-Cys-His-Met [18]. The fact that in such an oxidation-sensitive sequence only His and none of the Met or Cys residues were modified demonstrates the chemical selectivity of the site-specific oxidation mechanism. Amino acid analysis and sequencing of radioactive labeled peptides indicated the conversion of His into Asp and Asn [18] although later 2-oxo-His (structure 1; Scheme 1) was identified as an additional reaction product of the MCO of glutamine synthetase [19]. Mechanistic studies indicated the importance of H_2O_2 for product formation; however, H_2O_2 alone did not convert glutamine synthetase into an inactive form [20]. These results suggested the involvement of Fenton type reactions, generating hydroxyl radicals or their metal-bound equivalents (reactions 1 and 2). In fact, Dean et al. demonstrated that the exposure of proteins to hydroxyl radicals converted His into Asp [21], a product also present in hydrolysates of oxidized glutamine synthetase.



$$M^{n+} + H_2O_2 \rightarrow M^{(n+1)+} + HO^- + HO^{\bullet} \quad (1)$$

$$M^{n+} + H_2O_2 \rightarrow M^{(n+1)}(OH) + HO^- \quad (2)$$

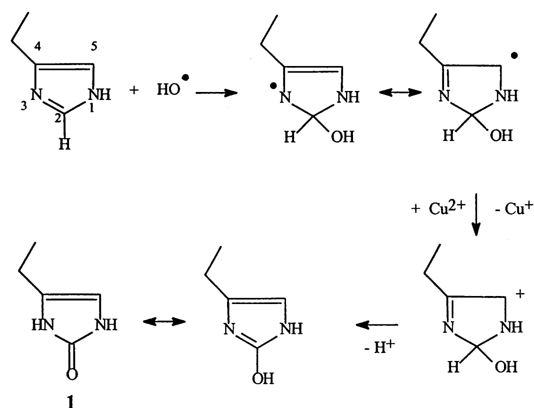
An interesting observation was made when Uchida and Kawakishi studied the copper-catalyzed oxidation of small His-containing peptides or N-benzoyl-His. The exposure to Cu^{2+} and H_2O_2, resulted in an efficient conversion of the peptide His residues into Asn, Asp, aspartylurea, and formylasparagine [22]. Only in the additional presence of ascorbate did the oxidation process also yield 2-oxo-His [13,23–26]. However, ascorbate was not a requirement for 2-oxo-His formation during the incubation of Cu,Zn SOD with H_2O_2 [9]. This suggests that His oxidation is controlled, in part, by the sequence and structure of a protein as well as the geometry of the protein/peptide–metal complex. Further support for this hypothesis will be presented below. Based on the observations presented above, the mechanisms of 2-oxo-His formation in proteins as well as the parameters controlling His oxidation warrant further investigation. Here, we will discuss mechanistic investigations of the MCO of His in human growth hormone. This protein is characterized by a well-defined metal-binding site containing two His residues located in helix I, His_{18} and His_{21} [27].

3. Metal-catalyzed oxidation of His in hGH

The incubation of 18 μM hGH in 10 mM air-saturated sodium phosphate, pH 7.4, with 10 μM Cu^{2+} and 100 μM ascorbate resulted in a rapid ca. 80–90% loss of native protein within 60 min, as assessed by ion-exchange chromatography [15]. Tryptic mapping in combination with either electrospray or MALDI-TOF mass spectrometry revealed the predominant loss of two fragments containing His_{18} and His_{21}, respectively. In contrast, the tryptic fragment containing His_{151}, which is not part of the metal-binding site, did not reveal any significant change. These results were confirmed by NMR where the C2 protons of the imidazole systems were conveniently monitored. His_{18} and His_{21} were converted with efficiencies of nearly 100 and 50%, respectively, to 2-oxo-His. Such high efficiencies had previously not been observed with other proteins such as glutamine synthetase, Cu,Zn SOD, enolase, and trypsin where reported efficiencies were on the order of $\leq 12\%$ [19]. Provided that these low yields of 2-oxo-His reported in Ref. [19] are not experimental artefacts, it appears that some properties of hGH favor the metal-catalyzed formation of 2-oxo-His, for example the particular geometry of the metal-binding site. The oxidation of His in hGH required the intermediary presence of H_2O_2 and protein-bound Cu^+. Furthermore, when reactions were carried out in $^{16}O_2$ and $H_2^{18}O$, no incorporation of ^{18}O into 2-oxo-His was detected. All these observations are in accord with a Fenton-type mechanism where site-specifically generated hydroxyl radicals (or their metal-bound equivalents) convert His to 2-oxo-His. This mechanism derives support from the fact that, at sufficiently high concentrations ($\geq 28\%$, v/v), added 1-propanol can inhibit the oxidation of His. Moreover, when 1-propanol-d7 is added instead of 1-propanol-h7, the protection is two-fold less efficient, indicating a kinetic isotope effect for this competition system. This observation is consistent with the fact that 1-propanol has an effect on hGH conformation [28], and may insert into the metal-binding site where it could scavenge site-specifically generated hydroxyl radicals [15]. In fact, kinetic isotope effects for the reaction of hydroxyl radicals with protonated versus deuterated alcohols are on the order of 2.0 [29], i.e. on the order of the observed difference in hGH protection by 1-propanol-h7 and 1-propanol-d7.

In its simplest form, the mechanism of 2-oxo-His formation requires the addition of $HO^\bullet$ at the C-2 position of the imidazole ring, as displayed in Scheme 1 below (here, the His residue is shown with the 'pyrrole nitrogen' as N-1). Calculations have shown that, among all $HO^\bullet$-adducts to a substituted imidazole system, the C-2 adduct is the energetically lowest lying species [31]. The latter is expected to reduce Cu^{2+} to Cu^+, followed by deprotonation to yield 2-oxo-His.

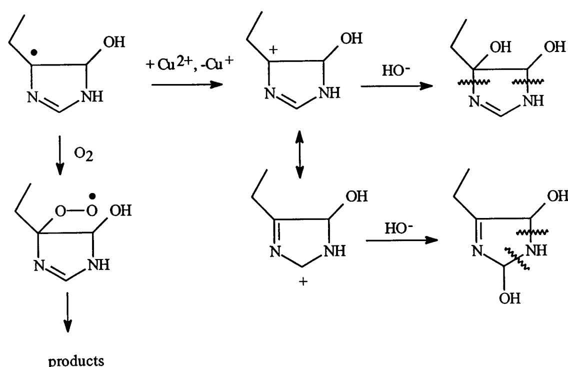
However, certain differences of this proposed mechanism to physical–chemical studies on the reaction of $HO^\bullet$ with imidazole and substituted imidazoles should be noted. Electron spin resonance studies have demonstrated that $HO^\bullet$ effi-



Scheme 1.

ciently adds to both the C2 and the C5 position of imidazole even when the C5 position carries an additional substituent such as a carboxylate group [30]. More recent experiments have concluded that addition occurs predominantly at C-5 [30]. As shown in Scheme 2, there is no simple pathway for a direct conversion of the imidazole C5-OH adduct into 2-oxo-His. It can be expected that C5-OH adducts either reduce Cu^{2+} or add molecular oxygen, ultimately resulting in the fragmentation of the imidazole system.

However, one possible alternative is provided by the known propensity of hydroxyl radical adducts of imidazole to eliminate water [31]. Normally, this reaction requires alkaline pH values [31,32]; however, added transition metals as well as the possibility of occurring within the interior of a protein, where basic residues may be close, could affect water elimination of hydroxyl radical

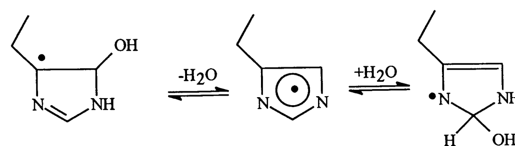


Scheme 2.

adducts of protein-bound imidazole rings. If water elimination were a reversible process, then an initial C5-OH adduct may convert into a C2-OH adduct through the processes displayed in Scheme 3.

We note that hydroxyl radicals may also add at the C4 position. The relative ratio of addition at C-5 versus C-4 may further depend on the fractions of His residue, especially in a protein, existing with N-7 as the pyrole versus pyrimidine nitrogen. However, as for the C5-OH adduct, the C4-OH adduct has no direct possibility of converting into 2-oxo-His unless reversible water elimination–readdition converts the C4-OH adduct into the C2-OH adduct.

In hGH, especially His_{18} is quantitatively converted to 2-oxo-His, suggesting either a stoichiometric addition of $HO^•$ at the C2 position or an efficient water elimination–readdition mechanism such as displayed in Scheme 3. In contrast His_{21} shows some reaction products other than 2-oxo-His, which may originate from an initial addition of $HO^•$ at C4 and/or C5. Several factors may be responsible for these differences between two His residues of the same protein on one hand, and between metal-catalyzed oxidation of a protein compared to reactions of freely diffusable hydroxyl radicals with imidazole derivatives in solution on the other hand. (1) His oxidation in hGH is metal-catalyzed and occurs with a His residue which is simultaneously involved in metal-binding. This fact may change the reactivity of the different positions of the imidazole ring towards $HO^•$. We note, however, that generally $HO^•$ reacts quite unselectively. Thus, the exclusive reaction of $HO^•$ with C2 based on a chemical reactivity of the different ring positions of the imidazole system would still be somewhat unusual. (2) The particular geometry of the metal-binding site may sterically favor the addition of $HO^•$ at the C2 position.



Scheme 3.

(3) No free but only metal-complexed hydroxyl radicals are formed, which may show different reactivities towards the individual positions of the His residues in hGH. (4) Water elimination–readdition mechanisms such as shown in Scheme 3 occur with different efficiencies depending on the location of a His residue within the protein structure.

At present we cannot decide to what extent each of these parameters potentially affects His oxidation in hGH and other proteins. However, the fact that the particular sequence around the His residues plays an important role in their reactivities derives support from additional experimental observations. We prepared the individual His-containing tryptic fragments of hGH and subjected them separately to MCO under conditions similar to those applied to the intact hGH. Both the His_{18}- and His_{21}-containing fragments did not suffer any degradation. This result is in contrast to other studies where small His-containing peptides underwent considerable oxidation, even to 2-oxo-His, when exposed to Cu^{2+} and ascorbate [13,23–26,32,33]. Thus, sequence and conformation are important parameters controlling the metal-catalyzed oxidation of His. Further studies with a series of proteins need to show whether general predictions on the efficiency and product patterns of His oxidation will be possible based on protein sequence and conformation.

Acknowledgements

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