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Oxidation of recombinant methionyl human granulocyte colony stimulating factor

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Abstract

The oxidation of methionine residues in recombinant methionyl human granulocyte colony stimulating factor with hydrogen peroxide has been investigated. Kinetic data of the oxidation were obtained by using reversed phase-high performance liquid chromatography. The stability-indicating capability of this system was confirmed with micellar electrokinetic capillary chromatography. In the pH range 1.9–7.5, the k_{obs} value for the oxidation process is constant. Above pH 7.5, k_{obs} tends to increase with increasing pH. In the pH range 1.9–11.8, four oxidation products were detected in RP-HPLC. Mass spectrometric analysis revealed that one mono-, one di- and two trioxidation products were formed. Using the cyanogen bromide cleavage method the nature of the oxidation products was determined. The mono-oxidation product is the protein with Met^{121} oxidized, while the dioxidation product has oxidized Met^{121} and Met^{126} residues. The trioxidation products are the proteins with Met^{121}, Met^{126} and Met^{137} or Met^{0}, Met^{121} and Met^{126} oxidized.

Keywords: Recombinant methionyl human granulocyte colony stimulating factor; Methionine residues; Neutropenia; Oxidation

1. Introduction

Recombinant methionyl human granulocyte colony stimulating factor (*r*-metHuG-CSF) is successfully applied in the prophylactic treatment of neutropenia [1–4]. *r*-metHuG-CSF is a hydrophobic protein consisting of 175 amino acids (Fig. 1) and differs from endogenous G-CSF by the virtue of an additional N-terminal methionine residue and the absence of *O*-glycosylation. Its isoelectric point (pI) is 6.2 [1,5]. 2D and 3D NMR analysis of ^{15}N- and ^{13}C-labelled *r*-metHuG-CSF showed that the compound consisted of four helices: residues 11–39 (helix A), residues 71–91 (helix B), residues 100–123 (helix C) and residues 143–172 (helix D) [6,7].

The overall chemical instability of *r*-metHuG-CSF involves degradation reactions like deamidation, reduction, oxidation and hydrolysis, taking

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place at various amino acid residues in the molecule. The nature and extent of the reactions are strongly influenced by the degradation conditions.

In *r*-metHuG-CSF the amino acid residues susceptible to oxidation are His (5 residues), Tyr (3), Trp (2), Met (4), Phe (5) and Cys (1 free Cys residue). Oxidation of His can lead to the formation of 2-oxo-His and a ring-opened residue which can be oxidized further to Asn. [8,9]. Tyr can be oxidized to a di-Tyr residue with the consequence of formation of intra- or intermolecular bonds. Oxidation of Trp residues leads to the formation of several products like *N*-formylkynurenin (the main product), oxindolylalanine and 5-OH-Trp. These products can be oxidized further to dioxindolylalanine, kynurenine and 3-OH-Trp, respectively [10–12]. Met oxidation gives rise to the formation of Met sulfoxides or Met sulfones [13–15]. Formation of *o*-Tyr is due to the oxidation of Phe residues [16–18]. Oxidation of Cys leads to the formation of disulfides with the formation of intra- and intermolecular bonds as a consequence [8,19]. Also formation of a sulfonic acid is possible [20]. The oxidation behaviour of *r*-metHuG-CSF in vitro and in vivo has not been studied. However, knowledge of the degradation of this protein may enable the optimization of formulation and storage of the drug.

In this paper qualitative and quantitative aspects of the oxidation of *r*-metHuG-CSF with hydrogen peroxide are presented.

2. Materials and methods

2.1. Chemicals

Neupogen® vials, 1 ml containing 300 μg *r*-metHuG-CSF, 0.59 mg acetate, 50 mg mannitol and 0.04 mg polysorbate 80 (pH 4) in sterile water for injection, was kindly donated by Amgen Inc. (Thousand Oaks, CA, USA). All other chemicals used are of analytical grade and deionized water is used throughout the study. Nitrogen gas is purified by passing through a column of activated copper followed by passage through a washing bottle containing a solution of 4 mM methyl viologen, 0.1 M pyrophosphate, 0.1 M EDTA and 40 mM proflavine with a pH 8.0 adjusted with 5 M hydrochloric acid.

2.2. Spectrometric analyses

UV spectrophotometric analysis is performed on a Hitachi Spectrophotometer Model 100-60 (Hitachi, Tokyo, Japan). Scanning speed is 60 nm min^{-1} within the range 320–240 nm.

Spectrofluorometric analysis is performed on a SFM 25 Spectrofluorometer (Kontron Instruments, Zürich, Switzerland). Using an excitation wavelength of 278 nm emission spectra are recorded within the range 400–300 nm with scanning speed 60 nm min^{-1}.

During oxidation of *r*-metHuG-CSF, by mixing 200 μl 10% hydrogen peroxide with 600 μl Neupogen®, spectra are recorded at regular time intervals.

2.3. Reversed-phase high performance liquid chromatography (RP-HPLC)

The gradient RP-HPLC system consists of a Gynkotek Model 480 pump (gradient controller), a Gynkotek Model 300 CS pump and an Applied Biosystems 785A Programmable Absorbance Detector (all from Separations, H.I. Ambacht, The Netherlands), a Model U6K Injector (Waters Associates, Milford, MA, USA) and a Phenomenex W-Porex 5 C4 300 Å, 150 × 4.6 mm column (Bester, Amstelveen, The Netherlands). Column

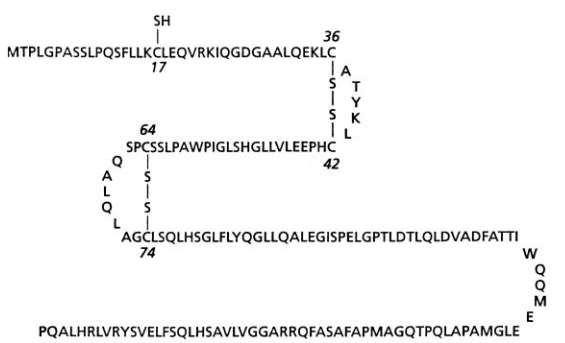

Fig. 1. Amino acid sequence of recombinant methionyl human granulocyte colony stimulating factor.

temperature is maintained at 50°C. For the kinetic studies the mobile phases consisted of A: aqueous 10 mM perchloric acid and 100 mM sodium perchlorate (pH 2), and B: 85% acetonitrile in water (w/w) containing 10 mM perchloric acid and 100 mM sodium perchlorate (pH 2). Separation is achieved using a linear gradient from 61 to 69% mobile phase B in 16 min followed by a 2-min isocratic elution of 69% mobile phase B after which it returns to 61% mobile phase B within 1 min. The injection volume is 10 μl, the flow is 1.0 ml min^{-1} and the detection is performed at 205 nm.

For the analysis of samples originating from the CNBr cleavage studies the following gradient is used: 5% B to 15% B in 5.5 min, in 2 min from 15% B to 40% B, in 15.5 min from 40% B to 80% B and in 2 min back to 5% B. The injected volume is 25 μl, the flow is 1.0 ml min^{-1} and the detection is performed at 205 nm.

2.4. Liquid chromatography-mass spectrometry (LC-MS)

For LC-MS experiments of the oxidation products the mobile phases consisted of C: 0.1% trifluoroacetic acid (TFA) in deionized water (pH 2), and D: 0.1% TFA in 85% acetonitrile in water (w/w) in a linear gradient ranging from 44 to 53% mobile phase D in 12 min, followed by a 2-min isocratic elution of 53% mobile phase D and return to 44% mobile phase D in 1 min. The flow was 0.2 ml min^{-1}. Column temperature was maintained at 50°C.

For LC-MS experiments of the CNBr digest, the mobile phases consisted of E: 0.1% trifluoroacetic acid (TFA) in deionized water (pH 2), and F: 0.1% TFA in 95% acetonitrile in water (w/w) in a linear gradient ranging from 0 to 25% mobile phase F in 5 min, followed by a linear gradient from 25 to 48% mobile phase F in 2 min. Next the gradient runs from 48 to 95% mobile phase F in 13 min followed by 3 min isocratic elution of 95% mobile phase F and back to 0% mobile phase F in 1 min. The flow was 0.2 ml min^{-1}. Column temperature was maintained at 50°C.

MS detection is performed using a VG Platform Benchtop LC-MS (Fisons Instruments, Altricham, UK). An electrospray interface is used to ionize the molecules (positive ion mode). The nebulizing gas has a flow of 25 l h^{-1}, the drying gas has a flow of 300 l h^{-1}. The applied voltage to the capillary is 3.4 kV, and a low cone voltage (22.0 V) is applied to prevent extensive fragmentation. The MS is calibrated from 166 to 1060 Da with a mixture of horse heart myoglobine (multiple charged) and peptides (in the range from 166 to 754 Da).

2.5. Data acquisition

Data analysis for RP-HPLC is performed using GynkoSoft software, version 4.12 (Separations, H.I. Ambacht, The Netherlands). MS data acquisition is carried out using MassLynx software, version 2.0 MS (Fisons Instruments, Altricham, UK).

2.6. Oxidation conditions

For the oxidation of *r*-metHuG-CSF the following conditions are used: Neupogen® is diluted with modified Britton-Robinson buffer [21], set at a pH in the range from 1.9 to 11.8 with sodium hydroxide, to an initial *r*-metHuG-CSF concentration of 27 μg ml^{-1} for the kinetic studies. All reactions are carried out in sealed vials at 0°C (melting ice). The H_2O_2 concentration in the samples is 0.11% (v/v).

For LC-MS experiments a stock solution of the oxidation is used. This stock solution is prepared by mixing 500 μl *r*-metHuG-CSF (300 μg ml^{-1}) with 250 μl H_2O_2 (0.3%, v/v) and allowing to generate at least four oxidation products; this takes approximately 30 min at 0°C. These solutions are centrifuged in Nanosep™ 10K Microconcentrators (Filtron, Terheijden, The Netherlands), the concentrate then is washed with deionized water three times to a final volume of approximately 50 μl. This is supplemented to 400 μl with deionized water to a *r*-metHuG-CSF concentration of 375 μg ml^{-1}.

2.7. Cyanogen bromide cleavage

Cleavage is carried out at pH 1 with *r*-metHuG-CSF or its oxidation products (approximately 270 μg ml^{-1}) and cyanogen bromide (CNBr) (500-fold excess with respect to the Met residues) at 80°C for 25 min. Cleavage using these conditions gives results similar to the cleavage under standard conditions (500-fold excess CNBr, ambient temperature, pH 1, 24 h) [22].

2.8. Identification of oxidation products

Using the CNBr cleavage assay the nature of the oxidation products can be determined. Oxidation products are separated on RP-HPLC using TFA in the mobile phase. Fractions are collected and mannitol is added in a concentration similar to the Neupogen® preparation. The fractions are freeze dried. The samples are reconstituted in water to a concentration comparable to that of the original sample. This sample is subjected to the CNBr cleavage assay.

3. Results and discussion

3.1. Stability-indicating capability

The stock solution, which contains at least four oxidation products and no native *r*-metHuG-CSF, is analyzed with RP-HPLC (Fig. 2). The experiment is repeated with native *r*-metHuG-CSF and an oxidized stock solution spiked with native *r*-metHuG-CSF.

In both techniques the oxidation products elute/migrate with different velocities from the native *r*-metHuG-CSF. These data indicate that the RP-HPLC system used is appropriate for kinetic studies. Since RP-HPLC shows better reproducibility in peak height compared to MECC the former technique is used to analyze and quantitate *r*-metHuG-CSF in degradation samples. There is no clear reason for the poorer reproducibility in MECC measurements. There are no indications that, during RP-HPLC analysis, the parent compound or its oxidation products degrade.

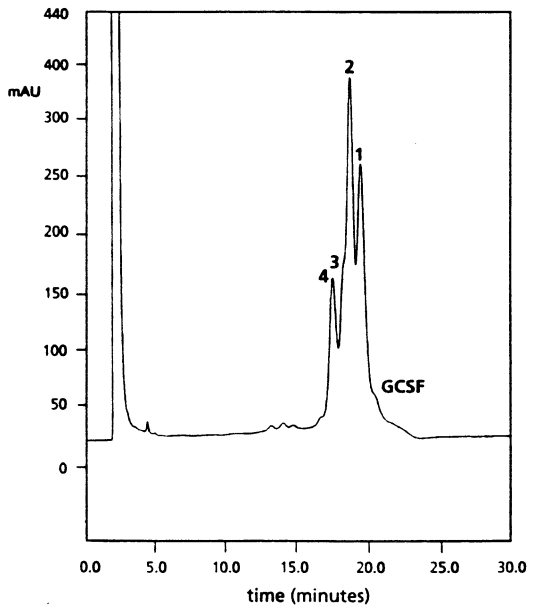

Fig. 2. RP-HPLC chromatogram of oxidized *r*-metHuG-CSF (GCSF). Peaks 1–4 are oxidation products. For conditions see text.

3.2. Kinetic studies

The kinetic data show that the degradation of *r*-metHuG-CSF is a (pseudo)-first order process. The standard deviation in k_{obs} for the oxidation reaction of *r*-metHuG-CSF is 0.02×10^{-4} s^{-1} (pH 6.5, $n = 4$) with an average k_{obs} of 1.19×10^{-4} s^{-1}. This standard deviation of 1.7% is in the usual order of magnitude for k_{obs} measurements.

The (pseudo)-first order degradation of *r*-metHuG-CSF has been investigated in the pH region 1.9–11.8. All degradations were carried out in two-fold. The reported values for k_{obs} are average values. In Fig. 3, the pH–log k_{obs} profile for the oxidation of *r*-metHuG-CSF with hydrogen peroxide is depicted. In the pH range 1.85–7.5, log k_{obs} for the oxidation reaction remains constant. From pH 7.5 up to pH 11.0 log k_{obs} tends to increase. These results indicate that there is no proton influence of protons on the k_{obs} value of the oxidation reaction while hydroxyl ions have only a slight influence, if any. The slight increase in oxidation velocity may very well be due to a pH-induced change in tertiary structure in the

region where Met^{121}, Met^{126} and Met^{137} are located. The CD loop (residue 124–142) in *r*-metHuG-CSF is more flexible than the rest of the molecule [6,7]. Therefore, Met residues might be better accessible in the higher pH-region, due to an overall change in charge since the pI of *r*-metHuG-CSF is 6.2 [5]. The chromatographic nature of the oxidation products of *r*-metHuG-CSF formed remains the same in the whole pH region investigated.

3.3. Spectrometric analyses

The UV spectra of oxidized samples and the spectrum of parent *r*-metHuG-CSF are identical. The absorbance at 280 nm does not decrease, indicating that the Trp residues are not subject to oxidation under these circumstances. This is consistent with literature data [23,24]. Spectrofluorometric analysis of the oxidation of *r*-metHuG-CSF shows no significant change in the emission spectra in the range 400–300 nm. These results indicate that Trp is not involved in oxidation [12].

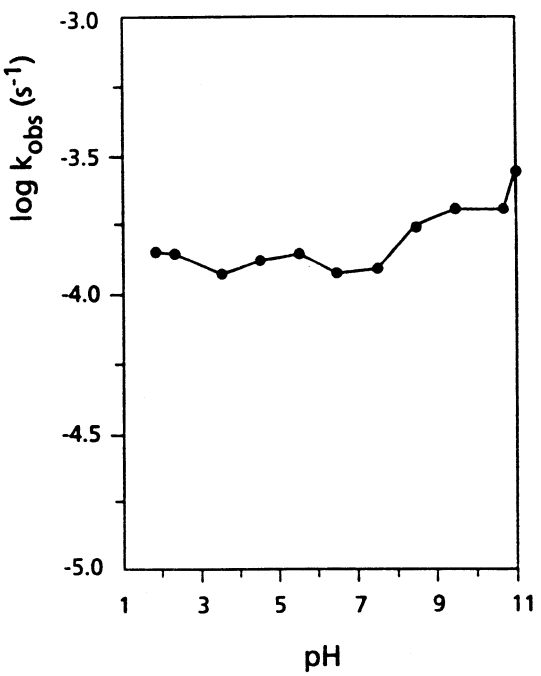

Fig. 3. pH–log k_{obs} profile of the oxidation of *r*-metHuG-CSF.

Table 1
Masses of *r*-metHuG-CSF and the oxidation products

Compound	Average mass ± standard deviation
r-metHuG-CSF	18 793 ± 1.5
Peak 1	18 808 ± 2.6
Peak 2	18 828 ± 2.6
Peak 3	18 842 ± 20
Peak 4	18 846 ± 4.5

RP-HPLC analysis of the samples shows that the parent compound has reacted completely and that all the oxidation products are formed in good detectable amounts. For the LC-MS measurements the separation of the various oxidation products turned out to be sufficient.

3.4. LC-MS

For the LC-MS identification of the various products, an oxidized stock solution of *r*-metHuG-CSF was used to ensure that the amounts of the various oxidation products were sufficient for accurate MS measurements.

Parent *r*-metHuG-CSF as well as the oxidized stock solution have been analyzed with the LC-MS system. The MS results are calculated from the m/z distributions of these peptides. The average values for m/z of *r*-metHuG-CSF and the oxidation products (peak 1–4, Fig. 2) are listed in Table 1. Considering the usual experimental error in m/z (4–6 Da) in LC-MS it may be justified to designate peak 1 as a monooxidation product, peak 2 a dioxidation product and peak 3 a trioxidation product. Although the difference in mass of peak 4 with the parent mass (53 a.m.u.) is more than 48 a.m.u. it is still plausible that 4 is also a trioxidation product as the difference is within the experimental error.

3.5. Identification of oxidation products

As stated before, peaks 1–4 are different oxidation products of *r*-metHuG-CSF. Using the CNBr cleavage assay the nature of the oxidation products can be revealed. CNBr cleaves the peptide backbone at the C-terminus of the Met

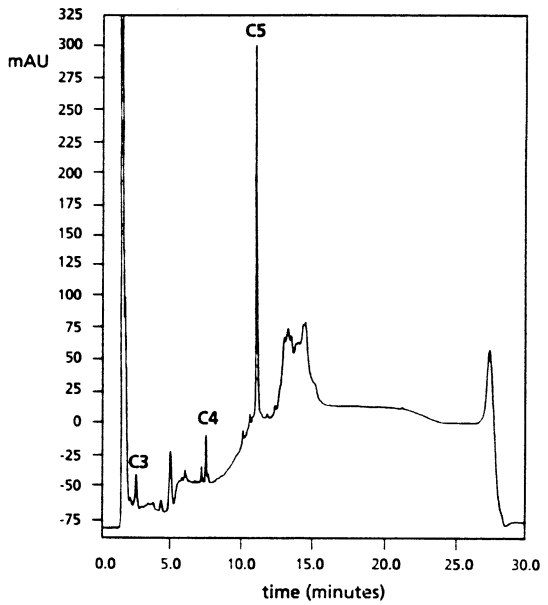

Fig. 4. RP-HPLC chromatogram of cyanogen bromide cleavage of *r*-metHuG-CSF. Fragments C3, C4 and C5 are Glu^{122}–Met^{126}, Ala^{127}–Met^{137} and Pro^{138}–Pro^{175}, respectively.

residue. After oxidation of the sulfur atom in Met to its sulfoxide CNBr cleavage does not take place any more [21]. Non-oxidized *r*-metHuG-CSF produces five fragments in a CNBr cleavage test. These fragments are C1: Met^{0}, C2: Thr^{1}–Met^{121}, C3: Glu^{122}–Met^{126}, C4: Ala^{127}–Met^{137} and C5: Pro^{138}–Pro^{175}. With LC-MS fragments C3, C4 and C5 can be detected. (Fig. 4, Table 2). Fragment C1 is the N-terminal Met which is, as a result of the CNBr cleavage, converted into homoserine lactone and elutes in the dead volume. Fragment C2 is not detected in LC-MS. Isolation of the product(s) eluting between approximately 12.5–16 min (Fig. 4) and consecutive injection into MS shows that no mass is detected with the m/z of fragment C2. Only a m/z of 13723 a.m.u. is detected which cannot be ascribed to an insufficient CNBr cleavage.

Isolated oxidation products are cleaved by CNBr. These CNBr digests are then chromatographically compared with the digest of the parent *r*-metHuG-CSF. Disappearance of fragments gives, in combination with the LC-MS data from the oxidation products, information about the nature of these products. Unexpectedly no new fragments are detected, possibly due to the same cause as the absence of fragment C2. Intense efforts to improve the LC-MS system failed up till now.

RP-HPLC analysis of the CNBr cleavage of the monooxidation product (peak 1) shows that fragments C4 and C5 are detectable while fragment C3 has disappeared, indicating that oxidation has only taken place at the Met^{121} residue. CNBr cleavage of the dioxidation product (peak 2) shows that the only detectable fragment is C5. This indicates that besides Met^{121} also the Met^{126} residue is oxidized. The trioxidation product peak 3 cleaved with CNBr shows no fragments C3, C4 and C5, indicating that Met^{121}, Met^{126} and Met^{137} are oxidized. The trioxidation product 4 shows, after CNBr cleavage, that fragment C5 is the only fragment detectable which indicates that Met^{137} is not oxidized whereas Met^{0}, Met^{121} and Met^{126} must be. Unfortunately, the argumentation for the structures of the oxidation products is based only on exclusion. Positive identification of the fragments formed with CNBr cleavage failed up till now.

A possible explanation for the fact that the oxidation starts with Met^{121} instead of Met^{0} can

Table 2
Fragments from CNBr cleavage

	C3 = Clu^{122}–Met^{126}	C4 = Ala^{127}–Met^{137}	C5 = Pro^{138}–Pro^{175}
r-metHuG-CSF	+	+	+
Peak 1 (monooxidation)	–	+	+
Peak 2 (dioxidation)	–	–	+
Peak 3 (trioxidation)	–	–	–
Peak 4 (trioxidation)	–	–	+

be that Met^0 is located on a hydrophobic part of the *r*-metHuG-CSF while Met^{121} is located in a hydrophilic part. The explanation that Met^{121} is located on a hydrophilic part of the molecule is supported by data from Hill et al. [6]. In this study the structural relationship between various growth factors is investigated. It appeared that helix C, where Met^{121} is located, is important for receptor recognition and therefore must be located on the outside of the molecule in order to be reachable.

4. Conclusions

The RP-HPLC method used to follow the oxidation is stability-indicating. The experimental error in k_{obs} determination is in the usual order of magnitude.

In the pH range 1.9–7.5, the $\log k_{obs}$ remains constant, above pH 7.5 the k_{obs} tends to increase slightly. This might be caused by a change in tertiary structure in such a way that the Met residues are better accessible, causing an increase in reactivity.

Oxidation of *r*-metHuG-CSF in the pH-range studied results in the formation of four oxidation products: one mono- and dioxidation product and two trioxidation products. From CNBr cleavage experiments the oxidation products have been identified. The monooxidation product is *r*-metHuG-CSF oxidized at the Met^{121} residue. The dioxidation product is *r*-metHuG-CSF oxidized at the Met^{121} and Met^{126} residues. The trioxidation products being formed are Met^{121}, Met^{126} and Met^{137} oxidized *r*-metHuG-CSF and Met^0, Met^{121} and Met^{126} oxidized *r*-metHuG-CSF. There are no signs of oxidation processes in other amino acid residues under these circumstances.

A hypothesis has been developed for the susceptibility of Met^{121} for oxidation compared to the other Met residues in *r*-metHuG-CSF, related to the activity of the Met^{121} site for receptor recognition.

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