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Reduction of Cys^{36}–Cys^{42} and Cys^{64}–Cys^{74} disulfide bonds in recombinant human granulocyte colony stimulating factor

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Abstract

The Cys^{36}–Cys^{42} and Cys^{64}–Cys^{74} disulfide bonds in recombinant methionyl human granulocyte colony-stimulating factor were reduced to sulfhydryls with dithiothreitol or mercury. Both reduction reactions are dependent on the pH. The reduction reaction with dithiothreitol increased in rate with increasing pH; between pH 7–9 and above pH 10.5 this increase was less than in other regions. These observations are explained by repulsive forces between dithiothreitol and regions in granulocyte colony-stimulating factor which intensify in these pH-regions. The hydroxyl catalysis causes the overall increase in k_{obs} in the pH-region studied. The reduction of the disulfides with mercury is, as could be expected from the Nernst equation for disulfide reduction, also pH dependent: the half-wave potential decreases with increasing pH as predicted by theory.

Keywords: Disulfide bonds; Granulocyte; Stimulating factor

1. Introduction

Recombinant methionyl human granulocyte colony-stimulating factor (r-metHuG-CSF) is successfully used 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 granulocyte colony-stimulating factor 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 consists of four helices: residues 14–40 (helix A), residues 74–90 (helix B), residues 105–121 (helix C) and residues 147–167 (helix D) [6]. The two disulfide bridges in r-metHuG-CSF both occur in the loop between helixes A and B [5–7]; one between Cys^{36} and Cys^{42}, the other between

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Cys^{64} and Cys^{74}. The free Cys residue at position 17 is present, in contrast to the other Cys groups, in the reduced (SH) form.

Lu et al. investigated the folding and oxidation of r-metHuG-CSF [7]. Their starting material was the fully reduced r-metHuG-CSF obtained from *Escherichia coli*. With copper (II) sulfate this product was oxidized and folded into its native state. With site-directed mutagenesis (particular cysteine residues were substituted by serine) the physiochemical properties and biological activity of intermediates in the oxidation reaction were investigated.

In the overall chemical instability of r-metHuG-CSF several degradation reactions like reduction, oxidation and deamidation play a role. These degradation reactions most likely influence the pharmacological activity of the compound by changing its polarity and/or lipophilicity. Especially in the case of reduction of the disulfide bridges is expected to change dramatically the tertiary structure of r-metHuG-CSF with the probability of changing its bioactive properties. It is therefore appropriate to investigate the role of reduction of these disulfide bridges as part of a survey of the over-all degradation process of r-metHuG-GSF.

In this paper some qualitative and quantitative aspects of the chemical and electrochemical reduction of the disulfide bonds in r-metHuG-CSF are presented.

For the reduction of disulfide bonds various methods are available. Examples are the reductive reactions of the disulfides with sodium hydride, tri–n–butyl phosphine and dithiothreitol. Also electrochemical reduction at a mercury electrode can produce sulfhydryl groups out of a disulfide [8]. The reductions take place via different reaction mechanisms: sodium hydride, tri–n–butyl phosphine and dithiothreitol donate their electrons via the formation of intermediates [8]. These intermediates are sulfur groups adducted with the reducing agent. Donation of electrons by mercury proceeds via an electrochemical mechanism.

2. Materials and methods

2.1. Chemicals

Neupogen®, 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 were of analytical grade and deionized water was used throughout the study. Nitrogen gas was purified by passing through a column of activated

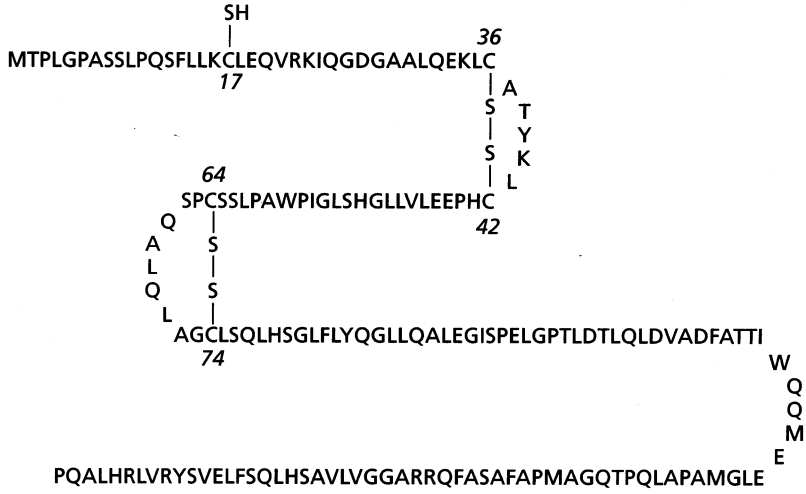

Fig. 1. Amino acid sequence of r-metHuG-CSF.

copper followed by passage through a washing bottle containing a solution of 4 mM methyl viologen, 0.1 M pyrophosphate, 0.1 M edathamil, 40 μM proflavine pH 8.0 adjusted with 5 M hydrochloric acid [9]. This elaborate treatement of the nitrogen gas is necessary to remove all the oxygen which is present in small amounts in the nitrogen used.

2.2. Square-wave voltammetry

Square-wave voltammetry (SWV) was carried out using an Autolab Potentiostat (Eco Chemie, Utrecht, The Netherlands) and a Model 303 Polarographic Detector (EG&G, Nieuwegein, The Netherlands) with a static mercury dropping electrode (SMDE), a Ag/AgCl/KCl (3 M) reference electrode and a platinum auxiliary electrode, controlled by GPES software version 3.3 (Eco Chemie). During 300 s an accumulation potential (E_{ac}) of 170 mV was applied. The potential scan was carried out from 0 to -1.5 V with a square-wave frequency of 50 Hz, a step potential (ΔE) of 1.98 mV and a square-wave amplitude of 20 mV. The experiments were carried out in Britton–Robinson buffer consisting of 0.04 M acetate, 0.04 M phosphate and 0.04 M borate. The pH is adjusted with 1 M sodium hydroxide to values in the range of 1.9–11.8.

2.3. Reversed-phase high performance liquid chromatography

The gradient Reversed-phase high performance liquid chromatography (RP-HPLC) system consisted 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). For the kinetic studies the mobile phases consisted of A: aqueous 10 mM perchloric acid and 100 mM sodium perchlorate (pH 2) and B: 70% acetonitrile in water (w/w) containing 10 mM perchloric acid and 100 mM sodium perchlorate (pH 2). Separation was achieved using a linear gradient from 59 to 71% mobile phase B in 12 min followed by a 3 min isocratic elution of 71% mobile phase B after which it returns to 59% mobile phase B within 1 min. The injection volume was 10–25 μl, the flow was 1.0 ml min^{-1} and detection was performed at 205 nm.

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

For LC-MS experiments 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 54 to 66% mobile phase D in 12 min, followed by a 3 min isocratic elution of 66% mobile phase D after which it returns to 54% mobile phase D in 1 min. The flow was reduced from 1.0 to 0.1 ml min^{-1} prior to component elution.

MS detection was performed using a VG Platform Benchtop LC-MS (Fisons Instruments, Altricham, UK). An electrospray interface was used to ionize the molecules (positive ion mode). The nebulizing gas had a flow of 25 l h^{-1}, the drying gas had a flow of 300 l h^{-1}. The applied voltage to the capillary was 3.4 kV, and a low cone voltage (22.0 V) was applied to prevent extensive fragmentation. The MS was 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 was performed using GynkoSoft software, version 4.12 (Separations, H.I. Ambacht, The Netherlands). MS data acquisition was carried out using MassLynx software, version 2.0 MS (Fisons Instruments, Altricham, UK).

2.6. Reduction/oxidation conditions

For the reduction of the disulfide bonds the following conditions were used: Neupogen® was diluted with Britton–Robinson buffer set at a pH

in the range from 1.9 to 11.8 with sodium hydroxide. The initial r-metHuG-CSF concentration ranged from 30 μg ml^{-1} for the kinetic studies to 270 μg ml^{-1} for the SWV experiments. All reactions were carried out under nitrogen atmosphere in a sealed vial at ambient temperature or 37°C. The concentration dithiothreitol (DTT) used in the kinetic studies was 2 mM.

For the reduction of r-metHuG-CSF at mercury and consecutive RP-HPLC analysis the following conditions were used: Neupogen® was diluted to a r-metHuG-CSF concentration of 100 μg ml^{-1} with Britton–Robinson buffer (pH 9.4). This solution was electrolyzed with a mercury pool as working electrode, a Ag/AgCl/KCl (3 M) as reference electrode and platinum as an auxiliary electrode. At ambient temperature a voltage of −1.0 V was applied. Aliquots of the reaction mixtures were analyzed with RP-HPLC.

For the oxidation of free thiols to disulfides in (partially) reduced r-metHuG-CSF the sample was treated with 40 μM copper (II) sulfate in 2% sodium dodecyl sulfate (SDS) [7]. Prior to oxidation, r-metHuG-CSF was separated from the excess DTT and other buffer components using a Sephadex G-10 column to avoid the possibility that thiols like DTT are involved in the formation of disulfide bonds.

3. Results and discussion

SWV was used to examine the pH influence on the half-wave potential ($E_{1/2}$) of the reduction of the disulfide bridges in r-metHuG-CSF. In Fig. 2 it can be seen that with increasing pH the $E_{1/2}$ decreases. The half reaction for the reduction of a disulfide bond shows that the reduction is dependent on the proton concentration

$$PS-SP + 2H^+ + 2e^- \leftrightarrow 2PSH \quad (1)$$

According to Friedman [8] the Nernst equation for this half-reaction is:

$$E = E^\circ + \frac{RT}{2F} \ln \frac{[PSSP]}{[PSH]^2} + \frac{RT}{F} \ln [H^+] \quad (2)$$

where E is the electrode potential, E° the standard reduction potential, R is the gas constant, T the absolute temperature and F the Faraday constant. In Fig. 2, the (linear) relationship between $E_{1/2}$ and pH is shown. From the slope of the linear relationship it can be concluded that in the reduction the number of electrons and the number of protons are equal, fully in agreement with the third term in the right-hand side of Eq. (2).

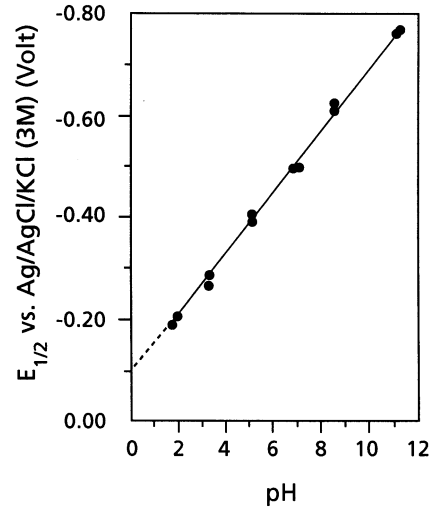

Fig. 2. Half-wave potential of the disulfides in r-metHuG-CSF as a function of the pH. $E_{1/2} = -0.062\ pH - 0.081$ V, $r = 0.999$.

RP-HPLC analysis of r-metHuG-CSF treated with DTT shows, that besides the parent compound, three other products are present (Fig. 3A). When the reduction process is followed in time, the peak height of the parent compound (peak 1) decreases following (pseudo-) first order kinetics while peaks 2 and 3 increase in height until they reach a maximum, after which they decrease in height. Peak 4 increases continuously until the peak of the parent compound as well as peaks 2 and 3 have fully disappeared (Fig. 4). During the reduction the area under the curve of peaks 1–4 corresponds with about 100%. This indicates that peaks 2 and 3 can be considered as intermediates with peak 4 as the fully reduced end-product. When the reduction is carried out at a mercury electrode only peak 4 is detectable besides the r-metHuG-CSF peak (Fig. 3B). The signals of both peaks decrease rapidly probably due to the high affinity of mercury for sulfur groups result-

ing in the formation of a complex between mercury and r-metHuG-CSF, which strongly adsorbs to mercury, causing a rapid decrease in the overall UV absorbance in the solution.

LC-MS of r-metHuG-CSF shows a m/z ratio of 18 801 Da ($\pm$4 Da), calculated from the m/z ratio distribution given in Fig. 5. The m/z ratios of the peaks 2, 3 and 4 are equal to the mass of the parent compound (Fig. 5) within the experimental error. With MS as detection technique it is not possible to show which of the disulfide bonds is reduced.

During isolation of peak 4 over Sephadex G-10 under ambient conditions the parent compound peak as well as peaks 2 and 3 are formed again. When copper (II) sulfate is added the parent compound also reappears almost quantitatively, together with small quantities of compounds 2 and 3 demonstrating the chemical reversibility of this redox reaction.

Kinetic studies of the reduction of the Cys^{36}–Cys^{42} and the Cys^{64}–Cys^{74} disulfide bonds of r-metHuG-CSF show that the disappearance of the parent compound follows (pseudo-) first-order kinetics.

In Fig. 6 log k_{obs} for the reduction of r-metHuG-CSF with DTT is plotted against the pH. Within the pH-range studied, a hydroxyl-mediated catalysis is observed: k_{obs} increases with increasing pH. In the pH-region 5–7 the slope of the pH–log k_{obs} profile is +1.1, in the pH region 9.5–10.5 the slope is +0.9 indicating specific hydroxyl catalysis in these pH-regions: the mercaptide anion of DTT is the species that reacts with the disulfide bond [8]. The profile exhibits an inflection between pH 7 and 9.5 while it levels out above pH 10.5. The inflections are probably due to increasing electrostatic repulsions between the reaction sites of r-metHuG-CSF and DTT. In the direct proximity of the disulfide bond Cys^{64}–Cys^{74} amino acid residues with charged side chains under the pH conditions studied are, however, absent, in contrast to the amino acid residues in the neighbourhood of the Cys^{36}–Cys^{42} disulfide bond. Here three Glu ($pK_a = 4.3$, positions 33, 45 and 46), two Lys ($pK_a = 10.5$, positions 34 and 40), one Tyr ($pK_a = 10.2$, position 39) and one His ($pK_a = 6.0$, position 43) residue are present (Fig. 1). However, these pK_a values are only an indication, they can alter in the peptide itself. DTT has two pK_a values: $pK_{a,1} = 8.3$ and $pK_{a,2} = 9.5$. Between pH 7 and 9.5 there is an inflection in the pH–log k_{obs} profile. In this pH region DTT becomes negatively charged due to deprotonating of the sulhydryl groups, and therefore approaches the negatively charged r-metHuG-CSF more

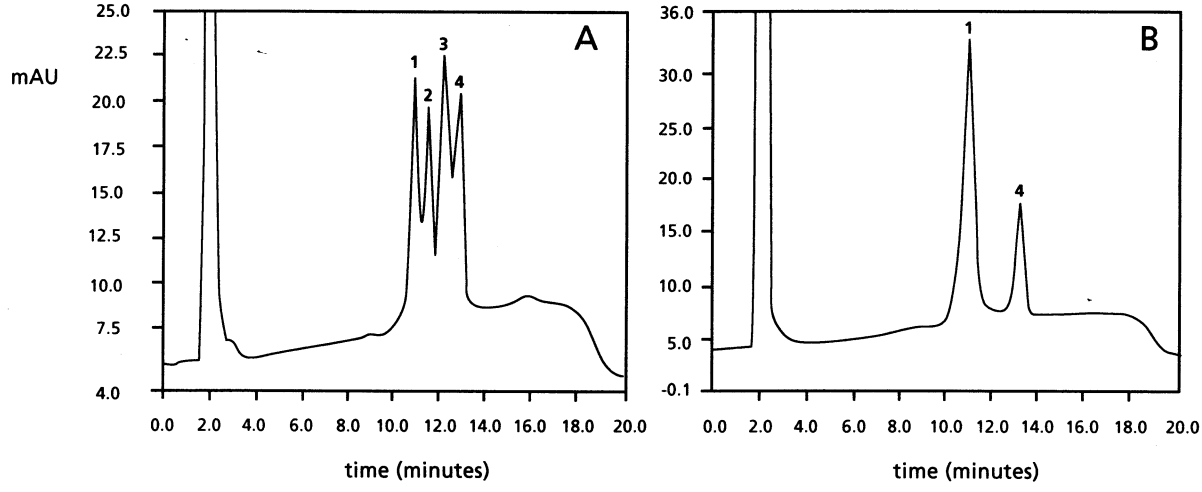

Fig. 3. (A) RP-HPLC chromatogram of r-metHuG-CSF reduced with DTT at two half-lives. (B) RP-HPLC chromatogram of r-metHuG-CSF reduced with mercury for 10 min. Peak 1: r-metHuG-CSF, peaks 2 and 3: intermediates in case where only one disulfide bond is reduced. Peak 4: fully reduced r-metHuG-CSF.

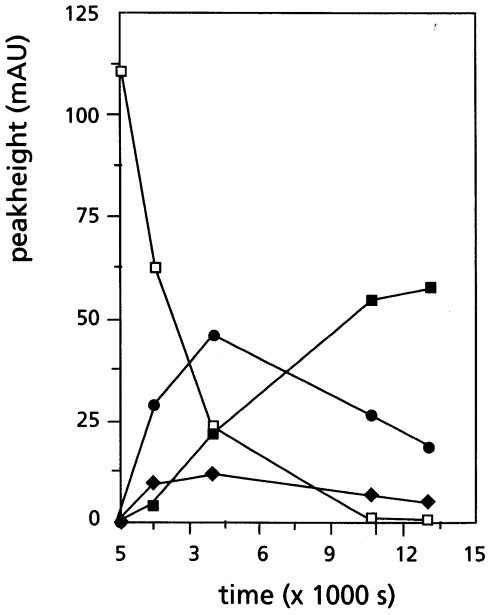

Fig. 4. Height of peak 1–4 plotted against the time. Decrease of r-metHuG-CSF (□) and formation of product 2 (●), product 3 (◆) and the fully reduced r-metHuG-CSF(product 4, ■). Reduction was carried out under nitrogen at ambient temperature in a Britton–Robinson buffer (pH = 7) containing 30 μg ml^{-1} r-metHuG-CSF and 2 mmol l^{-1} DTT.

difficultly. This is in accordance to the theory of Van der Houwen et al. [10] which describes the influence of ligands on pH–log k_{obs} profiles. The second inflection in the pH–log k_{obs} profile is above pH 10.5. This is caused by the increasing negative charge in the Cys^{36}–Cys^{42} region of r-metHuG-CSF (Table 1) and the formation of thiolate di-anion causing increased electrostatic repulsion of the thiolate di-anion of DTT.

On the basis of the LC-MS measurements of the intermediates in the chromatograms of the reduction process in the pH-region studied conclusive structural evidence can not be given. Below pH 9, however, there is a distinct preference for the formation of peak 2, between pH 9 and 10.8 peaks 2 and 3 are formed in equal quantities, while above pH 11 the formation of peak 3 prevails.

When chromatography is carried out with the system used as described by Lu et al. [7] similar chromatograms are obtained. Lu et al. identified the peaks by the use of site-directed mutagenesis where the Cys residues were replaced by Ser residues. Peak 3 is the intermediate where Cys^{17}, Cys^{64} and Cys^{74} are in reduced state with the Cys^{36}–Cys^{42} disulfide bond still existing. These results imply that peak 2 must be the intermediate with free thiols at Cys^{17}, Cys^{36}, Cys^{42} and a disulfide bond between Cys^{64}–Cys^{74}. The observations are in accordance with the shift in stability of intermediates, formed in the reduction of r-metHuG-CSF by DTT, which occurs between pH 9 and 11.

At pH values below 9 the net charge of the region between the 33rd and the 46th amino acid residues is slightly negative to neutral. At higher pH the Lys and Tyr residues are being deprotonated yielding a higher net negative charge. Negatively charged DTT may thus be electrostatically repulsed in this region (pK_a values mentioned before). The preference for reducing Cys^{64}–Cys^{74} with a relatively higher rate than the Cys^{36}–Cys^{42} disulfide at higher pH values seems therefore assumptive. The preference for the reduction of Cys^{36}–Cys^{42} at low pH values is probably due to the 3D folding of the protein. The Cys^{64}–Cys^{74} region is rather hydrophobic, the Cys^{36}–Cys^{42} region hydrophilic, which may influence the accessibility of the different regions for hydrophylic reagents.

4. Conclusions

In contrast to the research of Lu et al. [7] this study describes the qualitative and quantitative aspects on the reduction of the disulfide bonds in r-metHuG-CSF.Reduction of the Cys^{36}–Cys^{42} and Cys^{64}–Cys^{74} disulfide bond in r-metHuG-CSF is a reversible process: reduction can be carried out with DTT as well as at a mercury electrode, while reoxidation is possible with copper (II) sulfate. Reduction with DTT yields, besides the fully reduced product, two intermediates whereas reduction at mercury only gives the fully reduced r-metHuG-CSF. Parent r-metHuG-CSF, partially and fully reduced products formed all have, within experimental errors, masses of 18.80 kDa.

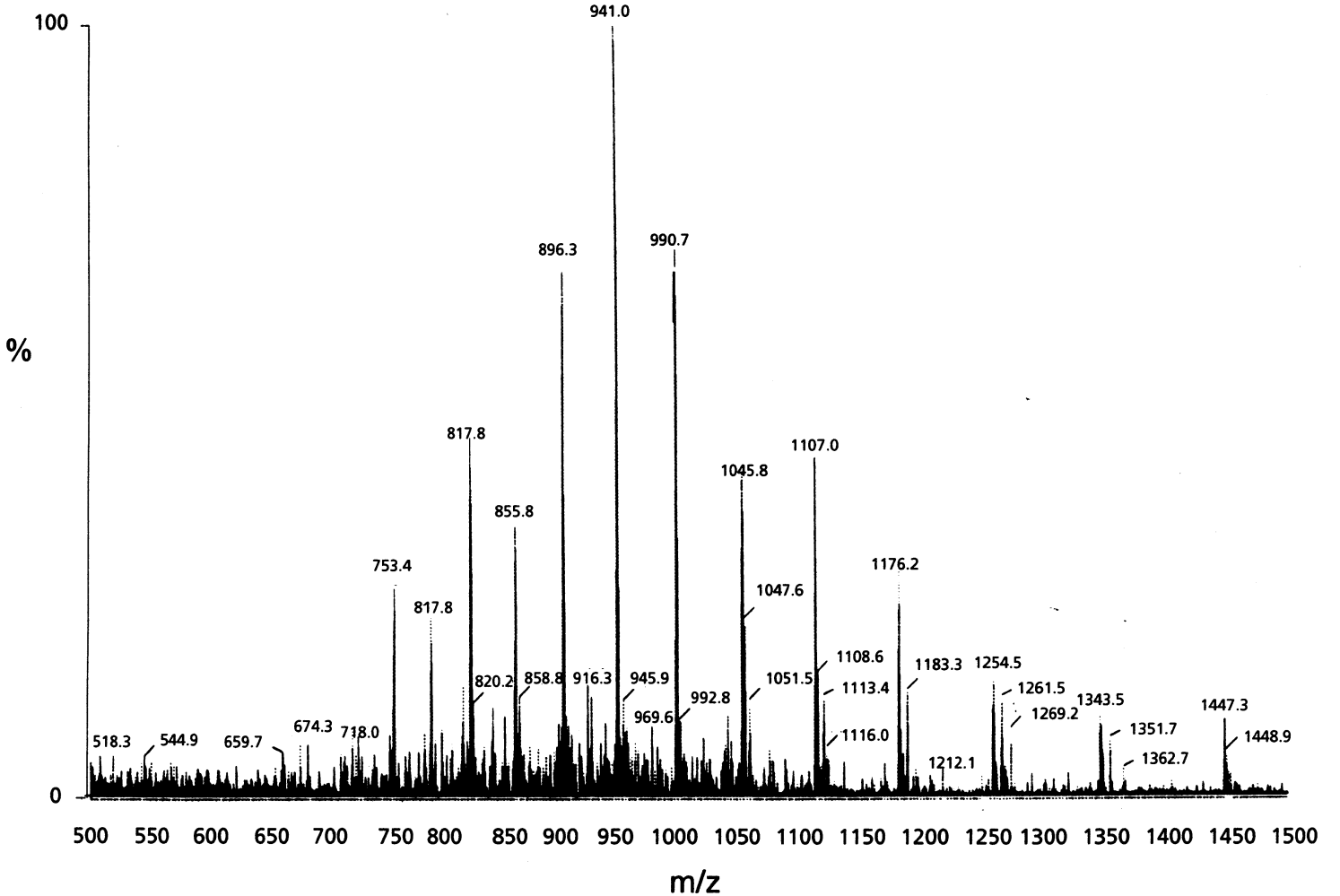

Fig. 5. MS spectrum of the multiple charged r-metHuG-CSF.

Table 1
Charges of r-metHuG-CSF between the 33rd and the 46th amino acid at different pH values

Amino acid	Number of residues	Side chain (pK_a)	Charge			
			pH = 6	pH = 8	pH = 10	pH = 12
Glu (E)	3	COOH (4.3)	3−	3−	3−	3−
Lys (K)	2	NH_2 (10.5)	2+	2+	~1+	0
Tyr (Y)	1	OH (10.2)	0	0	1/2−	1−
His (H)	1	NH (6.0)	1/2+	0	0	0
Net charge			1/2−	1−	21/2−	4−

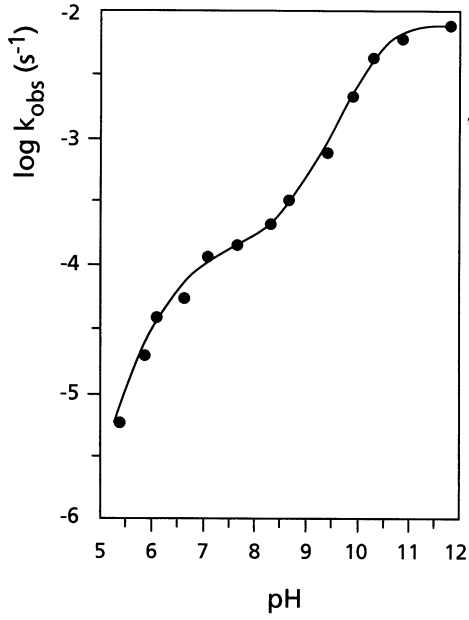

Fig. 6. pH–log k_{obs} profile for the reduction of the Cys^{36}–Cys^{42} and Cys^{64}–Cys^{74} disulfide reduction in r-metHuG-CSF with DTT at ambient temperature.

Square-wave voltammetric measurements show that the relationship between pH and half wave potential for the reduction of r-metHuG-CSF is linear and in accordance with the Nernst Eq. (2). This relationship indicates that the number of protons and electrons involved in the redox reaction are equal.

Kinetic studies on the reduction of the disulfide bonds in r-metHuG-CSF with DTT show that within the pH-region 5–12 the k_{obs} increases linearly between pH 5–7 and pH 9.5–10.5, the slope of the curve being approximately +1 indicating that specific hydroxyl catalysis occurs. Inflections in the pH–log k_{obs} profile are explained by increasing electrostatic repulsive forces between the reacting species.

Reductions carried out in the pH region below 9 show that the formation of peak 2, presumably r-metHuG-CSF with a disulfide bridge between Cys^{64}–Cys^{74} but with free CysSH functions at positions 36 and 42, prevails. Above pH 11 the formation of peak 3, probably r-metHuG-CSF with one disulfide bridge between Cys^{36}–Cys^{42} but with free CysSH residues at positions 64 and 74, is most abundant. In the pH region 9–11 peaks 2 and 3 are formed at similar rates.

Looking in this way to the reduction of peptides in general probably a good prediction can be made for the order of formation and nature of the obtained reduction products. However, this is not the aim of this study and therefore needs more experimental evidence to justify the predictive value of this approach.

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