Oxidation of Human Insulin-like Growth Factor I in Formulation Studies: Kinetics of Methionine Oxidation in Aqueous Solution and in Solid State

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Purpose. The aim of this work was to study the kinetics of oxidation of methionine in human Insulin-like Growth Factor I (hIGF-I)[1] in aqueous solution and in the solid state by the aid of quantification of oxygen.

Methods. The oxidized form of hIGF-I was characterized by tryptic peptide analysis, RP-HPLC and FAB-MS and quantified by RP-HPLC. The oxygen content was quantified polarographically by a Clark-type electrode.

Results. Second-order kinetics with respect to amount of protein and dissolved oxygen was found to be appropriate for the oxidation of methionine in hIGF-I. The rate constants ranged from 1 to 280 M^{-1} $month^{-1}$ and had an activation energy of 95 (+/− 4) kJ/mole. Light exposure, storage temperature and oxygen content were found to have a considerable impact on the oxidation rates. No significant difference in reaction rates was found for the oxidation of hIGF-I in aqueous solution or in the solid state. A method for decreasing the oxygen content in aqueous solution without purging is described.

Conclusions. Polarographic quantification of dissolved oxygen makes it possible to establish the kinetics for oxidation of proteins. The oxidation of methionine in hIGF-I appears to follow second-order kinetics.

KEY WORDS: hIGF-I; oxidation; methionine; HPLC; kinetics.

INTRODUCTION

Human Insulin-like Growth Factor I (hIGF-I) is a peptide hormone mediating the growth-promoting effect of human growth hormone. In the systemic circulation hIGF-I is bound to several specific binding proteins (1). Binding of hIGF-I to these binding proteins has been shown to increase the half-life of the protein considerably (2) and recently it was demonstrated that the binding proteins also stabilize the disulfide bonds of hIGF-I in vivo and thus maintain its correctly folded state (3).

Human IGF-I is a small globular protein containing 70 amino acids (4), see Figure 1. Its molecular weight is 7650 Dalton. Blundell et al., 1983 (4) have proposed the tertiary structure of hIGF-I on the basis of the known structure of the related protein insulin. The solution structure of hIGF-I has also been elucidated in NMR-studies (5,6). The tertiary structure suggested by Blundell was confirmed by these studies. The studies have revealed that hIGF-I in aqueous solution contains three alpha-helices, two relatively rigid (I and II) and one more flexible (III). Potential modification reactions include oxidation of a methionine residue (Met59), deamidation of the asparagine and glutamine residues (Asn26 and Gln15) and reduction of the three disulfide bridges (Cys6–Cys48, Cys47–Cys52 and Cys18–Cys61).

Early development work indicated that the protein, in the pH-range 3–7, was stable towards aggregation during storage and that it was reasonably stable towards other modification reactions. Above pH 7, however, aggregation occurred. Oxidation of the methionine residue appeared to be the major modification pathway, although several other modified products were detected.

The amino acid residues containing sulfur, i.e., methionine and cysteine can be oxidized. In methionine, the nucleophilic sulfur in the thioether group is oxidized to a sulfoxide. This oxidation is reversible and with an appropriate reducing agent the sulfoxide can be reduced back to its corresponding sulfide. Under more severe conditions the sulfoxide is additionally oxidized to a sulfone. This reaction is irreversible but occurs seldomly under conditions of interest to formulators. The dependence of methionine oxidation on oxygen content indicates that formation of an oxygen radical of some sort determines the rate of reaction. This dependence has been established for, e.g., human growth hormone (7). Numerous authors have reported dependence of oxidation on oxygen, but the difficulties to actually quantify the oxygen has so far limited the possibilities to perform kinetic calculations under pharmaceutically relevant conditions. Polarographic quantification of dissolved oxygen (e.g., 8, 9) can be made in small sample volumes with great accuracy.

The purpose of this study was to investigate the effect of oxygen content, temperature, light exposure, protein concentration, some radical scavengers and storage in aqueous solution or in solid state on the oxidation of the methionine in hIGF-I. Further, the purpose was also to determine the kinetics of the reaction.

MATERIALS AND METHODS

Materials

Yeast-derived recombinant hIGF-I (10) was purified by several chromatographic methods and was assayed by the developed RP-HPLC method (see below) and found to be at least 95% pure.

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ABBREVIATIONS: RP-HPLC = Reversed Phase High Performance Liquid Chromatography; hIGF-I = human Insulin-like Growth Factor I; Met59 = Methionine residue 59 in hIGF-I; Asn26 = Asparagine residue 26 in hIGF-I; Gln15 = Glutamine residue 15; Cys6-Cys48 = The disulfide bridge between the cysteine residues 6 and 48, FAB-MS = Fast Atom Bombardment Mass Spectometry, TFA = Trifluoroaceticacid.

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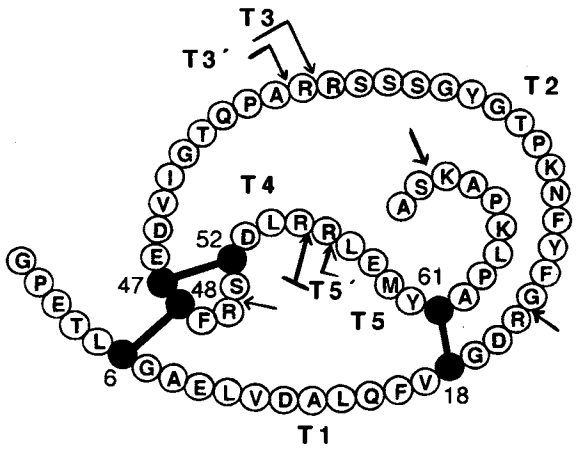

Fig. 1. Amino acid sequence of hIGF-I and disulfide bridges. Cleavage sites for trypsin digestion and the notation for peptides formed are indicated.

Trypsin, dithioerythritol, sodium dithionite, hydrogen peroxide, trifluoroacetic acid (TFA), bovine catalase, L-mannitol, sodium azide were purchased from Sigma and used as received. Glycerol, tert-butyl alcohol and ammonium bicarbonate were purchased from Merck and used as received. Acetonitrile was purchased from Lab-Scan and used as received. Propanesulfonic acid was purchased from Fluka and used as received. All other chemicals used complied with Ph. Eur. 2nd Ed.

Preparation of Samples

After purification the bulk solution was loaded on to a Sephadex G-50 column (Pharmacia LKB, Sweden) from which the protein was eluted with 50 mM sodium phosphate buffer, pH 6. The solutions were sterile filtered through 0.22 μm hydrophobic polyvinylidene difluoride membrane filters (Millipore Corp., USA). Portions of 1 mL were dispensed into sterile glass vials, made of type I borosilicate glass. The containers were sealed with bromobutyl rubber stoppers (Helvoet Pharma, Belgium).

To investigate the effect of the oxygen content a method for minimizing the amount of oxygen was developed (patent application to be published). Filled vials were placed in a sterilized pressure chamber. The temperature was lowered to +6°C, the vials were allowed to equilibrate and the pressure was lowered to 10 mbar. Dissolved oxygen was degassed at this low ambient pressure. The pressure was raised to atmospheric pressure with pure nitrogen gas (<5 ppm O_2). This procedure was repeated three times.

Samples to be lyophilized were frozen on the freeze-drier shelves at a temperature of −30°C. Drying was thereafter performed at +25°C and at 1.0 mbar for 13 hours. The vials were stoppered in the freeze-dryer with a head-space consisting of sterile filtered air (21% oxygen). The residual moisture content in the vials were quantified by loss on drying to be less than 1% (w/w).

Methods to Quantify Levels of Oxygen

The amount of dissolved oxygen was determined by a Clark-type oxygen mini-electrode (Yellow Springs Instruments, USA). The electrode was calibrated with distilled oxygen-free water (considered as 0%), containing sodium dithionite as an oxygen scavenger, and with distilled water freshly bubbled with oxygen gas (100% of maximum oxygen solubility). All measurements were performed at a controlled temperature of +25°C and in duplicates. The theoretical oxygen contents in the solutions at the temperatures studied were then calculated. It was assumed that the ratio of the oxygen content in the solution to the maximum oxygen solubility was constant. The maximum oxygen solubility values were determined in thermostated solutions after purging with pure oxygen gas for 30 minutes and are shown in figure 2. This assumption was considered appropriate due to the equilibrium between dissolved and gaseous oxygen. The equilibrium at a given temperature T can be presented as:

$$O_2(aq) = Q_2(g) * k_{O_2} - O_2(react.)$$

The term O_2(react.) represents the oxygen consumed by reactions and is in the experiments studied very small in relation to the other two amounts and can therefore be neglected. The solubility constant kO_2 increases as the temperature T is lowered, thus O_2(aq) will increase.

The amount of dissolved oxygen in the untreated samples ranged from 260 to 300 mmole/L and in the treated samples from 25 to 50 mmole/L. For the lyophilized samples the absolute amount of molecular oxygen was estimated to be 6.9 $*10^{-5}$ mole per vial, assuming that 1 mole of air had a volume of 22.4 liters at 1 atm (11).

Influence of Temperature and Light

To study the stability of hIGF-I in the different formulations, samples were stored in thermostated facilities at temperatures of 7°C (±1°C), 25°C (±1°C), 37°C (±1°C) and 50°C (±1°C). Samples were withdrawn for analysis at appropriate intervals. The samples were stored protected from light unless otherwise specified. Experiments for light sensitivity of hIGF-I were performed at 25°C (±1°C) and with an exposure of 1100 lux from an electric daylight lamp. This type of lamp emits light with wavelengths above 370 nm (12)

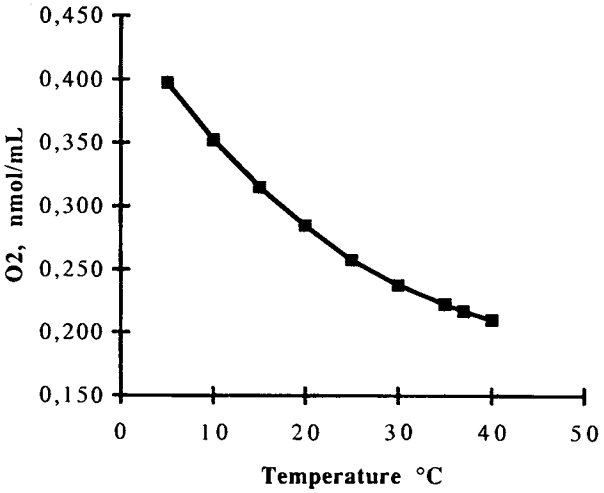

Fig. 2. Oxygen solubility in water at different temperatures.

Identification of Oxidized hIGF-I

Preparation of oxidized hIGF-I for characterization was performed by dissolving 600 μg of lyophilized hIGF-I in 0.1 mole/L ammonium bicarbonate to a volume of 0.5 mL. Then, 5 μL 30% hydrogen peroxide, known to preferentially oxidize methionine (13) was added. The solution was stored in an ice-bath for 2 hours and then bovine catalase was added to terminate the oxidation. The sample was kept at room temperature for 15 minutes to complete the reaction.

To 1.2 mg samples of hIGF-I and oxidized hIGF-I 40 μg trypsin was added and the samples were incubated for 3 hours at +37°C. Then, 1 mg dithioerythritol was added and the solutions were incubated for another 15 minutes. 10 μL trifluoroacetic acid (TFA) was added to adjust the pH. Separation was obtained by RP-HPLC on a column, Vydac C_{18}, 5 μm, 300 Å, 250 × 4.6 mm^3. Elution solutions were: (A) 0.01 mole/L sodium phosphate buffer, pH 2.0 and (B) 40% of A and 60% acetonitrile. The peptides were eluted with a linear gradient running from 92 to 59% of solution A and at the same time from 8 to 41% of solution B. The detection wavelength was 210 nm. After desalting the fragments were analyzed by fast atom bombardment mass spectrometry on a JEOL SX-102 mass spectrometer (JEOL, Japan) with xenon as collision gas. The ion source was equipped with a FAB-gun. A positive ion mass spectrum was recorded in the accumulation mode covering the mass range 0–2400 Dalton. The instrument was calibrated with cesium iodide prior to analysis of the sample. 2 μL of the solutions above was applied onto a FAB probe and dried in vacuum. The dried samples were then mixed with 0.7μL glycerol prior to analysis.

Quantification of Oxidized hIGF-I

Separation was obtained by RP-HPLC on a silica Bakerbond column, C_8, 5 μm, 300 Å, 250 × 4.6 mm^3. Elution was accomplished by changing the mobile phases, A and B, according to a gradient running from 64 to 38% of solution A in 20 minutes, then maintaining 38% A for 5 minutes and then a gradient running from 38 to 64% of A in 5 minutes. Solution B was changed simultaneously to add up to 100%. Solution A consisted of 0.02 mole/L sodium phosphate buffer, pH 2 and 0.01 mole/L propanesulfonic acid sodium salt in 10% aqueous acetonitrile. Solution B consisted of 0.02 mole/L sodium phosphate buffer, pH 2 and 0.01 mole/L propanesulfonic acid sodium salt in 50% aqueous acetonitrile. Detection was performed at 210 nm. Each sample was injected in duplicate. The percentages of oxidized and authentic hIGF-I in the samples were calculated from the relative areas in the chromatograms. Validation of the method was performed by injection of known amounts of authentic and oxidized hIGF-I.

Kinetic Modeling

A kinetic model fully describing the complex phases of oxidation reactions is difficult to propose. The oxidation can in a simple manner be described by the following model.

$$IGF + {}^1/_2O^2 \xrightarrow{k_{ox}} oxIGF \qquad (1)$$

Where IGF represents the oxidisable substance, O_2 molecular oxygen, oxIGF is oxidized hIGF-I and k_{ox} is the rate constant for the reaction. This reaction is supported by the stoichiometry found by Sysak et al. (14) for oxidation of methionine at pH 6 or below. From reaction (1) the following theoretical kinetic model for oxidation was derived.

$$\frac{d(oxIGF)}{dt} = k_{ox}*(IGF_{init} - oxIGF)*\left(O_{2init} - \frac{oxIGF}{2}\right) \qquad (2)$$

Equation (2) can be further integrated and summarized to:

$$kox*t = \frac{1}{\frac{IGF_{init}}{2} - O_{2init}} * \ln \frac{O_{2init}*(IGF_{init} - oxIGF))}{\left(IGF_{init}*\left(O_{2init} - \frac{oxIGF}{2}\right)\right)} \qquad (3)$$

where k_{ox} is the second-order reaction rate constant, t is the time in months, IGF_{init} and O_{2init} are the initial amounts of hIGF-I and O_2 respectively.

For comparison, the observed rate constants for total modification of hIGF-I during storage were also calculated. As the kinetics of the total modification were unknown, the observed rate constants for these reactions were assigned zero order.

The actual amounts of oxygen, oxidized or authentic hIGF-I in mole/litre were fitted into model 3 and plotted as functions of time. Second-order rate constants were then determined by calculating linear slopes in the figures. The observed zero-order rate constants for total modification of hIGF-I were calculated by plotting the moles of formed modified hIGF-I per total moles in the formulation (including excipients) as a function of the storage time in months. The residuals (R) for the calculated curve-fits were 0.7 for samples at 7°C, 0.9 for samples at 25°C and 1.0 for samples at 37°C, 50°C or light exposed samples.

RESULTS

Isolation and Characterization of Modifications of hIGF-I

Oxidized and authentic hIGF-I were digested by trypsin and the tryptic peptides were separated by RP-HPLC as described above. The peptides T5 and T5′ correspond to the peptides 56–68 and 57–68, respectively, both containing residue Met59. Only T5 and T5′ in the oxidized sample differ in retention times from the peptides obtained from authentic hIGF-I. Mass determination revealed a mass difference of 16 units in both peptides T5 and T5′ originating from oxidized and authentic hIGF-I, indicating addition of an oxygen to the methionine residue.

Kinetics of Methionine Oxidation in Aqueous Solution

Experiments were made with a solution consisting of 0.26 mM hIGF-I, 50 mM sodium phosphate buffer, pH 6, and 150 mM sodium chloride. The second-order rate constants for methionine oxidation (k_{ox}) and the observed zero-order rate constants for total modification of hIGF-I (k_{tot}) are displayed in table 1. An Arrhenius plot for the ln k_{ox} values against 1/T exhibited a linear relationship (not shown). The activation energy (E_a) for the oxidation of hIGF-I (Met59) was determined to be 95 (+/−4) kJ/mole. The ratio between the rate constants for oxidation and for total modification of hIGF-I appears to be constant as long as the samples are protected from light. When exposed to light the k_{ox}/k_{tot} ratio increases substantially, indicat-

Table 1. Second-order Rate Constants for Oxidation (k_{ox}) of hIGF-I (Met59) and Observed Zero-order Rate Constants for Total Modification (k_{tot}) of hIGF-I. Experiments Were Performed with Solutions of 0.26 mM hIGF-I, 50 mM Sodium Phosphate Buffer at pH 6 and 150 mM Sodium Chloride. The Rate Constants Given Are Calculated as Means from Data for 3 Different Batches. The Figures Represent the Means of the Experiments ± the Respective Standard Deviations

	Rate constants		
Temperature	k_{ox} M^{-1} $month^{-1}$	k_{tot} mole $month^{-1}$ $*10^{-5}$	k_{ox}/k_{tot} $*10^3$
7°C	1.1 (±0.3)	3.6 (±0.8)	30
25°C	10 (±1)	37 (±1)	30
37°C	57 (±6)	190 (±10)	30
25°C Light	280 (±200)	210 (±140)	130

ing that radical generation is increased. Light exposure clearly accelerates both oxidation as well as total modification. Methionine oxidation is the dominating modification pathway during light exposure.

Effect of Radical Scavengers

Addition of 10–300 mM mannitol, 100 mM tert-butyl alcohol or 100 mM sodium azide known to effectively scavenge hydroxyl or superoxide radicals respectively had no effect on the oxidation rates neither in darkness nor in light.

Influence of Composition, State, and Environmental Parameters on the Oxidation of Methionine

Protein Concentration

Rate constants were calculated for solutions with 0.26, 0.9 and 1.4 mM hIGF-I. The second-order rate constants for methionine oxidation (k_{ox}) and the observed zero-order rate constants for total modification (k_{tot}) at 7, 30 and 50°C are displayed in table 2. At 7° the rate constants for methionine oxidation appeared to be constant in relation to protein concentration while the rate constant at 30° and 50°C decreased with protein concentration. The rate constants for total modification increased with the concentration at all temperatures. This shows that at higher protein concentrations oxidation is decreased due to an increased ratio between protein molecules and oxygen radicals. This would indicate that the oxygen radicals are formed independently of the protein molecules. This could also be exemplified by the fact that addition of free L-methionine in excess decreases the rate of oxidation of methionine in a protein chain (15). The other modification reactions, however increase with protein concentration, possibly due to higher intermolecular interactions. This is supported by an observed higher degree of aggregation for higher protein concentrations (data not shown). The ratio of the methionine oxidation to the other modification reactions also decrease with the increasing protein concentration.

Oxygen

Deoxygenation of aqueous solutions is usually conveniently performed by purging with an inert gas such as nitrogen or argon. This method is however, of limited use for protein solutions due to their ability to foam. Degradation and modification of peptides and proteins can also be enhanced by the creation of new solution-gas interfaces and by the mechanical stress imposed during purging. A more suitable method to deoxygenate aqueous solutions of proteins was developed as described above. Oxidation of hIGF-I in phosphate buffer solutions with two amounts of dissolved oxygen are shown in figure 3. While the rate of oxidation is dependent on the amount of

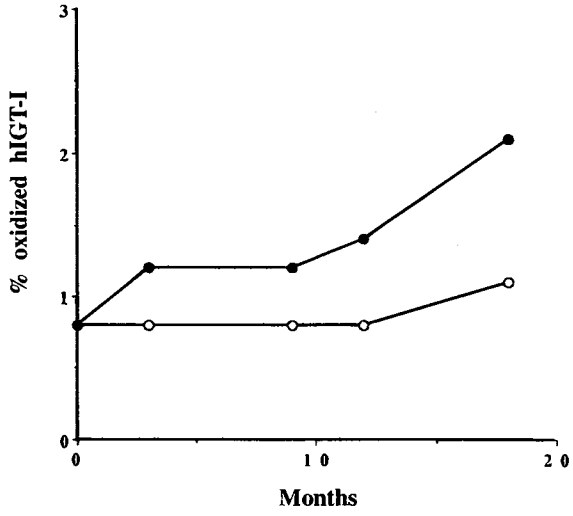

Fig. 3. Oxidation of hIGF-I (Met59) in solutions with different amounts of dissolved O_2 at 25°C. The oxygen content was 50 mmole O_2/L (open symbols) and 280 mmole O_2/L (filled symbols). The solutions contained 0.26 mM hIGF-I and 50 mM sodium phosphate, pH 6. The values presented reflect the amounts of oxidized hIGF-I as percentages of the amount of hIGF-I at time 0.

Table 2. Second-order Rate Constants for Oxidation (k_{ox}) of hIGF-I (Met59), Observed Zero-order Rate Constants for Total Modification (k_{tot}) of hIGF-I, and Ratio Between Oxidation and Total Modification at Different Protein Concentrations and Temperatures. The Protein Was Formulated in 50 mM Sodium Phosphate Buffer pH 6 and 150 mM Sodium Chloride. The Rate Constants at 50°C Were Not Available for the Samples with 1.4 mM

mM	k_{ox} M^{-1} $month^{-1}$			k_{tot} mole $month^{-1}$ $*10^{-5}$			k_{ox}/k_{tot} * 10^3		
hIGF-I	7°C	30°C	50°C	7°C	30°C	50°C	7°C	30°C	50°C
0.26	1	22	270	3	50	1000	3	40	30
0.9	0.6	20	30	10	100	2000	6	20	2
1.4	0.6	16	a	20	600	—	—	3	a

oxygen available, the rate constant is not. The modification of hIGF-I by other pathways was not affected by the amount of oxygen present.

Solid or Aqueous State

In table 3 the second-order rate constants for methionine oxidation (k_{ox}) and the observed zero-order rate constants for total modification (k_{tot}) in freeze-dried samples are shown. The samples were stored in vials with a headspace consisting of air, i.e. 21% oxygen. A comparison with the values for k_{ox} obtained for oxidation in aqueous solutions, table 1, shows that the oxidation rate appears to be similar in the temperature range 25°C to 30°C. The oxidation rate at 50°C however, equals the oxidation rate found in the aqueous solution at 37°C, indicating that at higher temperatures oxidation is somewhat slower in the solid state.

Note that the ratio of oxidation rate versus the total modification rate for hIGF-I in the solid state differs from the same ratio obtained for aqueous solutions, see table 1. In the solid preparation oxidation represents a much larger part of the total modification of the protein. The ratio k_{ox}/k_{tot} is larger for hIGF-I in the solid state than in solution. It is likely that other reaction pathways are retarded by the low total mobility in the solid state. The ratio is however temperature-dependent in the solid state as well and decreases with the temperature.

DISCUSSION

Previous reports on oxidation in aqueous solutions have often been intimidated by the decrease in oxygen solubility with temperature. By use of polarographic quantification of oxygen at several temperatures these problems were now solved and the kinetics of the oxidation of methionine could be determined. The formation of radicals is generally independent of temperature (16). As the oxidation of methionine increased with the temperature the reaction appears to be mainly determined by diffusion. Light had a clear impact on the oxidation rates. The oxidation rate increased by a factor 30 when exposed to light. In pulse-radiolysis experiments with methionine in aqueous solution (17) the oxidation was fast, e.g., 2.3×10^{10} M^{-1} s^{-1} or 9500 M^{-1} $month^{-1}$. The comparatively low rate constant, 280 M^{-1} $month^{-1}$, found in our study, at exposure to light at wavelengths above 370 nm indicates that generation of radicals during these conditions is low. The nature of the radicals involved in the oxidation of methionine in hIGF-I could not be determined from our experiments. However, regardless of the nature of the radicals involved, water is essential for the oxidation mechanism (7).

The observed rate constants for oxidation in the solid state were not substantially different from those of the aqueous solution. Diffusion of reactants is slow in the solid state as compared to in a solution. However, the thin water layer surrounding the solute molecules in the solid matrix may contain quantities of dissolved oxygen. Any residual oxygen in the solid would be trapped in a freeze-concentrate together with the amorphous protein, in considerable amounts. Townsend and co-workers (18) studied Ribonuclease A in the freeze-dried state and could quantify amounts of oxidation products. They hypothesized that the fixation of the protein and catalytic agents in close proximity in the solid matrix in fact would support radical formation and further oxidation.

Table 3. Second-order Rate Constants for Oxidation (k_{ox}) in hIGF-I (Met59) and Observed Zero-order Rate Constants for Total Modification (k_{tot}) in the Solid State. The Freeze-dried Formulations Consisted of 0.13 μmole hIGF-I, 50 μmole Sodium Phosphate, and Glycine or Glycine/Mannitol. The Amount of Oxygen in the Vials Was Estimated to 6.9 $*^{-5}$ Mole. The k_{tot} Values for Reactions at 7°C Varied too Much to Be Calculated Sufficiently Accurately. The Values Presented Represent Means of Data for Two Batches

	Rate constants		
Temperature	k_{ox} M^{-1} $month^{-1}$	k_{tot} mole $month^{-1}$ $*10^{-5}$	k_{ox}/k_{tot} $* 10^3$
7°C	1	—	—
30°C	14	5	280
50°C	56	39	140

CONCLUSIONS

By use of polarographic quantification of oxygen second-order kinetics of the oxidation of methionine in hIGF-I could be established. During conditions with free access to oxygen the oxidation rates are the same in the solid and in the aqueous state. Diffusion appears to determine the reaction. The precise mechanism of this reaction remains to be studied. Although oxidation of methionine is the major modification reaction with hIGF-I, it is comparatively slow. Out of the many solution and environmental conditions studied, exposure to light appears to possess the most profound effect on methionine oxidation in hIGF-I.

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