Oxidation of Human Insulin-Like Growth Factor I in Formulation Studies, II. Effects of Oxygen, Visible Light, and Phosphate on Methionine Oxidation in Aqueous Solution and Evaluation of Possible Mechanisms

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Purpose. The oxidation of methionine in human Insulin-like Growth Factor I (hIGF-I) in aqueous solution was studied with respect to oxygen, visible light and sodium phosphate.

Methods. Aqueous solutions of hIGF-I were prepared with different amounts of phosphate and dissolved oxygen. The solutions were stored either in darkness or exposed to artificial visible light. The oxidized hIGF-I was quantified by RP-HPLC. A two level full factorial experimental design, with two levels of each of the three factors studied, was used.

Results. Oxidation was found to be positively correlated with light, oxygen content and, interestingly, phosphate. The increasing effect of phosphate on the oxidation appears not to originate from metal contaminants. The influence of both oxygen and phosphate increased with time. The pH dependence of oxidation indicated the formation of a phosphorylated sulfonium ion as an oxidation intermediate. A significant interaction effect between phosphate and visible light suggested participation of radicals.

Conclusions. Factorial experiments provide a valuable tool when studying complex mechanisms with interacting factors. The oxidation of methionine in hIGF-I is significantly affected by light but also by the presence of phosphate buffer.

KEY WORDS: sulfur; protein; oxidation; chemometrics; phosphate.

INTRODUCTION

In a previous study (1), the oxidation of the methionine residue in human Insulin-like Growth Factor I (hIGF-I)[1] was investigated. It was concluded that light exposure had a large impact on the oxidation rate. Several other factors were also found to influence the kinetics, for instance physical state and temperature.

A conventional method for multifactor experimental designs is the "change-one-factor-at-a-time" method, which means that a single factor is varied while all other conditions are kept fixed. This method may lead to unreliable results and wrong conclusions (2). Factorial designs and multivariate regression methods can better deal with multifactor experiments. Factorial designs are robust, easy to understand, easy to apply and have found a widespread use (3). One of the most widely used methods of multivariate data analysis is Partial Least Squares (PLS) (4). PLS is used to optimize the relationship between two blocks of data (e.g. experimental variables and responses). The two blocks are modeled by separate models that are related to each other by a linear inner relationship. This means that only relevant information is extracted from the data, enabling substantial noise reduction.

Inorganic phosphate has been suggested to participate in the oxidation of methionine (5). Several authors have found a correlation between phosphate buffers and oxidation of both proteins and other compounds (e.g. 6,7). The general explanation is that it is trace-amounts of metal ion contaminants that participate in the oxidation reaction (e.g. 6,8). Since phosphate is a very common buffer for protein formulations, it would however be of importance to investigate if it *per se affects* the stability of proteins.

The purpose of our study has been to investigate the importance of oxygen, phosphate, visible light and their respective interactions on the oxidation of methionine in hIGF-I.

MATERIALS AND METHODS

Materials

In-house yeast-derived recombinant hIGF-I (figure 1) (9) was assayed by RP-HPLC (1) to be at least 99.5% pure.

Ultra pure MilliQ water (Millipore corp., USA) was used. Pro analysis grade disodium phosphate dodecahydrate, monosodium phosphate monohydrate, citric acid monohydrate and trisodium citrate dihydrate were purchased from Merck (USA). The chemicals for the RP-HPLC are described in (1). Chloride salts of Fe^{3+}, Ca^{2+}, Cu^{2+}, Mg^{2+} and Zn^{2+} were obtained from Merck (USA).

Statistical Analysis

The effects of two levels of intensity of visible light and two levels each of oxygen and phosphate contents on the oxidation of methionine in hIGF-I were studied in a full factorial design: 2^3, see table 1. This means that eight experiments were needed. Four additional experiments (two replicates with two identical experiments each) were carried out for determining the experimental variations. The response variables were percentages of oxidized hIGF-I at the different storage times.

In our regression model, the response variables were auto scaled (each variable scaled to unit variance) and the candidates for explanatory variables were linear and interaction terms of coded levels of light, oxygen and phosphate content.

PLS was used as modeling method and the optimal number of components has been determined by cross validation or by leverage correction (10). The experimental design and the evaluation of the data were performed by the CODEX software (SumIt System, Sweden).

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ABBREVATIONS: RP-HPLC = Reversed Phase High Performance Liquid Chromatography; hIGF-I = human Insulin-like Growth Factor I; Met59 = Methionine residue 59 in hIGF-I; PLS = Partial Least Squares.

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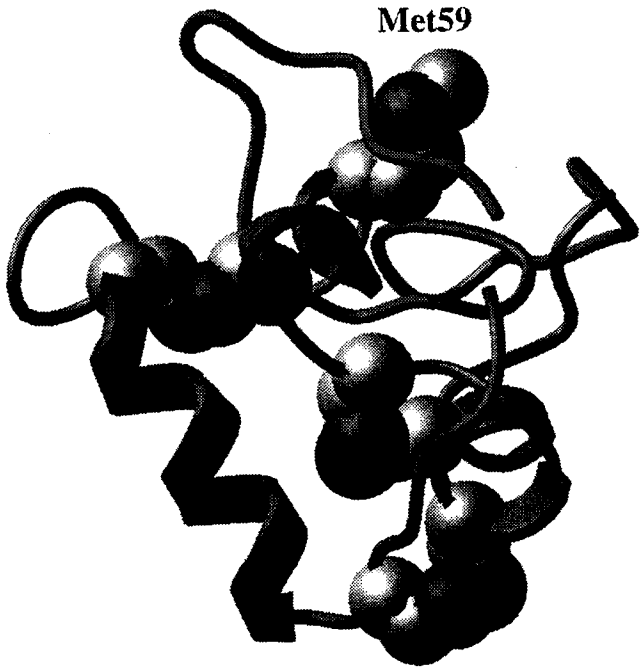

Fig. 1. The tertiary structure of hIGF-I with helices, disulfides and the methionine residue (19).

Preparation of Samples

Cleaning of the Vials

To ensure that no catalytic metal ions contaminated the system, a rigorous cleaning protocol was followed as described by Fyhr and Brodin (11).

Table 1. The Experimental Design Worksheet Containing the Different Factors, Their Respective Levels, and the Responses

FACTORS			RESPONSES % oxidized hIGF-I[c]					
Light[a]	Oxygen[b]	Phosphate (mM)	2 days	7 days	14 days	28 days	56 days	98 days
−1	−1	10			0.2		0.3	0.5
1	−1	100	0.3	0.8	1.3	2.2	4.0	7.1
−1	−1	10			0.2		0.4	0.5
−1	−1	100	0.1	0.2	0.2	0.6	0.6	0.7
1	1	100	0.5	1.0	2.0	3.1	9.0	10.0
1	1	100			2.5		6.3	17.1
−1	1	10	0.2	0.3	0.6	0.6	2.1	
−1	1	100	0.2	0.3	0.6	0.5	1.1	1.8
−1	−1	10	0.2	0.2	0.3	0.3	0.3	
1	1	100			2.2		5.5	
1	−1	10	0.4	0.8	0.8	1.2	3.5	
1	1	10	0.2	0.4	1.0	2.0	8.0	

[a] 1 denotes continuous exposure to visible light, −1 denotes total darkness.

[b] 1 denotes 300 mM O_2, −1 denotes less than 25 mM O_2.

[c] The values reflect the percentages of hIGF-I containing oxidized methionine after different storage times at 25°C.

Levels of hIGF-I and Phosphate

A hIGF-I bulk solution was divided into two parts which were mixed with buffer solutions to make formulations containing 0.13 mM of hIGF-I with either 10 or 100 mM sodium phosphate, pH 6.3.

Dispensing into Vials

Each of the two formulations were further divided into two parts which were handled either in oxygen-free atmosphere or in air (21% oxygen). All solutions were sterile filtered through 0.22 μm hydrophobic polyvinylidene difluoride membrane filters (Millipore Corp., USA), dispensed into borosilicate glass vials and stoppered with bromobutyl rubber stoppers (Helvoet Pharma, Belgium).

Quantification of Dissolved 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 containing sodium dithionite as oxygen scavenger, and with distilled water freshly bubbled with oxygen gas. All measurements were performed at a controlled temperature of 25°C (±0.5°C) and in duplicates. The amount of dissolved oxygen at the start of the study in the aerobic solutions was 300 mM and in the anaerobic solutions less than 25 mM.

Stability Testing

Storage Conditions

The samples were stored at a temperature of 25°C (±1°C) in facilities with a continuos exposure of 1100 lux from an electric day-light lamp. The samples to be stored protected from light were wrapped in aluminum foil and placed in positions close to the light-exposed samples in order to be stored under identical temperature conditions.

Quantification of Oxidized hIGF-I

A RP-HPLC method was used to determine the purity of the hIGF-I preparations and the amount of oxidized hIGF-I (1). Briefly, the separation was obtained on a silica Bakerbond column, C_8, 5 mm, 300 Å, 250 × 4.6 mm. Elution was accomplished by changing the mobile phases, A and B, in a gradient. Solution A consisted of 0.02 mole/L sodium phosphate buffer, pH 2, 0.01 mole/L propanesulfonic acid sodium salt in 10% acetonitrile. Solution B consisted of 0.02 mole/L sodium phosphate buffer, pH 2, 0.01 mole/L propanesulfonic acid sodium salt in 50% 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 by the relative areas in the chromatograms. A relative standard deviation of less than 3% was established for the quantification of oxidized hIGF-I. The accuracy of the method was tested by adding known amounts of oxidized hIGF-I and comparing the amounts found with the theoretical values. When the experimental values were plotted against the theoretical values the slope was found to be 1.09.

pH Measurements

The pH values of the solutions were recorded on a Metrohm 632 pH-meter (Switzerland) with an Orion combination electrode (Switzerland).

RESULTS AND DISCUSSION

Levels of Metal Contaminants

Trace amounts of metal contaminants, especially iron, might affect the oxidation. In a pre-experiment 0.15 ppm (w/w) of chloride salts of Fe^{3+}, Ca^{2+}, Cu^{2+}, Mg^{2+} and Zn^{2+} were added to solutions 0.13 mM hIGF-I and 50 mM sodium phosphate, pH 6.0. No effect of the added metals on the oxidation could be detected. However, when 1 ppm of the metals were added a significant increase in oxidation was noticed for Fe^{3+} only. The solutions used in the full-scale oxidation study were analyzed by atomic absorption spectroscopy and none of the above metal ions could be detected. The limit of detection for metals was 0.05 ppm.

PLS Modeling

A PLS model was constructed using experimental settings depicted in table 1. The model was expanded by inclusion of cross terms (interaction terms) of light vs. oxygen, light vs. phosphate and oxygen vs. phosphate. The number of significant PLS-components was determined by calculating the ratio between Prediction error sum of squares (PRESS), from the cross validation procedure and the Residual sum of squares (RSS) of the previous dimension. If their ratio is below one, the model performs better than a randomly chosen model. The model comprised two significant PLS-components, with a ratio of 0.59 resp 0.98 for components 1 and 2. These two components explain 86% of the variance of the response Y. A relative standard error of prediction of 21%, for the 6 responses, was obtained. Light was the factor which alone had the greatest influence on the model. This was revealed by a very large linear term and also by its synergism with phosphate (see figure 2). Both oxygen and phosphate had an impact but less pronounced than light.

Further, the influence of oxygen and phosphate increased with storage time (figures 2 and 3). Parallel to this increase, the magnitude of the interaction effects between light and phosphate and between oxygen and phosphate decreased with storage time.

Effect of Light Exposure on Oxidation of Methionine

The massive effect of irradiation on the oxidation was expected, but yet dramatic. Even though the bond energy for the O-O bond is low, only 35 kcal/mol, and light with wavelengths of up to 700 nm theoretically can generate oxygen radicals (12), the probability of oxygen radical formation decreases rapidly with increasing wavelength. The artificial daylight produced by a fluorescence lamp emits light at wavelengths above 370 nm (13) and is thus not expected to produce oxygen radicals in significant amounts. However, presence of other components can have an effect on the radical energetics (14).

The Effect of Oxygen on Oxidation of Methionine

The amount of oxygen had a small effect on the oxidation and the interaction between light and oxygen was insignificant throughout the whole experiment. The oxygen radicals are not primarily formed as a result of the irradiation, which is why it ought to be the nonexcited oxygen molecule that participates in the reaction. It thus appears that the light exposure would rather excite the methionine sulfur itself, as suggested above, which then would be available for oxidation by oxygen or phosphate.

The Effect of Phosphate on Oxidation of Methionine

The response surface plot at low oxygen content emphasizes the strong interaction between visible light and phosphate (figure 4). This indicates that light excites either the phosphate ions to an oxidizing radical or the sulfur in the methionine, which in a subsequent step reacts with phosphate. Whatever the exact mechanism, it appears to be independent of oxygen since the interaction effect is substantial even at a very low oxygen content.

Effects of Ionic Strength

To determine the possible effects of ionic strength, a control experiment was performed. Solutions of 0.13 mM hIGF-I and 5 mM sodium phosphate buffer, pH 6.0 with or without 145 mM sodium chloride were prepared. No significant differences in oxidation could be found for these solutions.

Mechanism of Phosphate Induced Oxidation of Methionine

Phosphate can affect oxidation reactions by a number of mechanisms.

Binding Metals

Phosphate may bind to divalent metals, preferentially Fe^{2+}, and stabilize the transfer of electrons from Fe^{2+} to molecular oxygen. However, this would be possible only during substoichiometric concentrations of phosphate to iron, in analogy with the effect of chelators such as EDTA (15). This means that a low level of phosphate but not a high level would increase oxidation. This is contrary to the experimental results obtained in our study.

Proton Donor Mechanism

Phosphate may act as a proton donor at acidic pH when present as $H_2PO_4^-$ (16), see scheme 1. $H_2PO_4^-$ ions are present in detectable amounts even at pH 6. The mechanism was suggested by Hiller after studies on free methionine. The $H_2PO_4^-$ ion would catalyze the oxidation by stabilizing a disulfur (Met_2) hydroxyl intermediate. This intermediate decomposes to oxidized methionine and methionine.

$$(R_2S)_2(OH)^\circ + H_2PO_4^- \Leftrightarrow (R_2S)_2^{+\circ} + HPO_4^{-2} + H_2O \rightarrow \text{products}$$

Scheme 1

However, the steric hindrance for forming the intermediate with a globular protein would be considerable. The pH in the solution with 10 mM phosphate decreased during light exposure which also makes this mechanism unlikely, as HPO_4^{-2} would be produced during the reaction and the pH

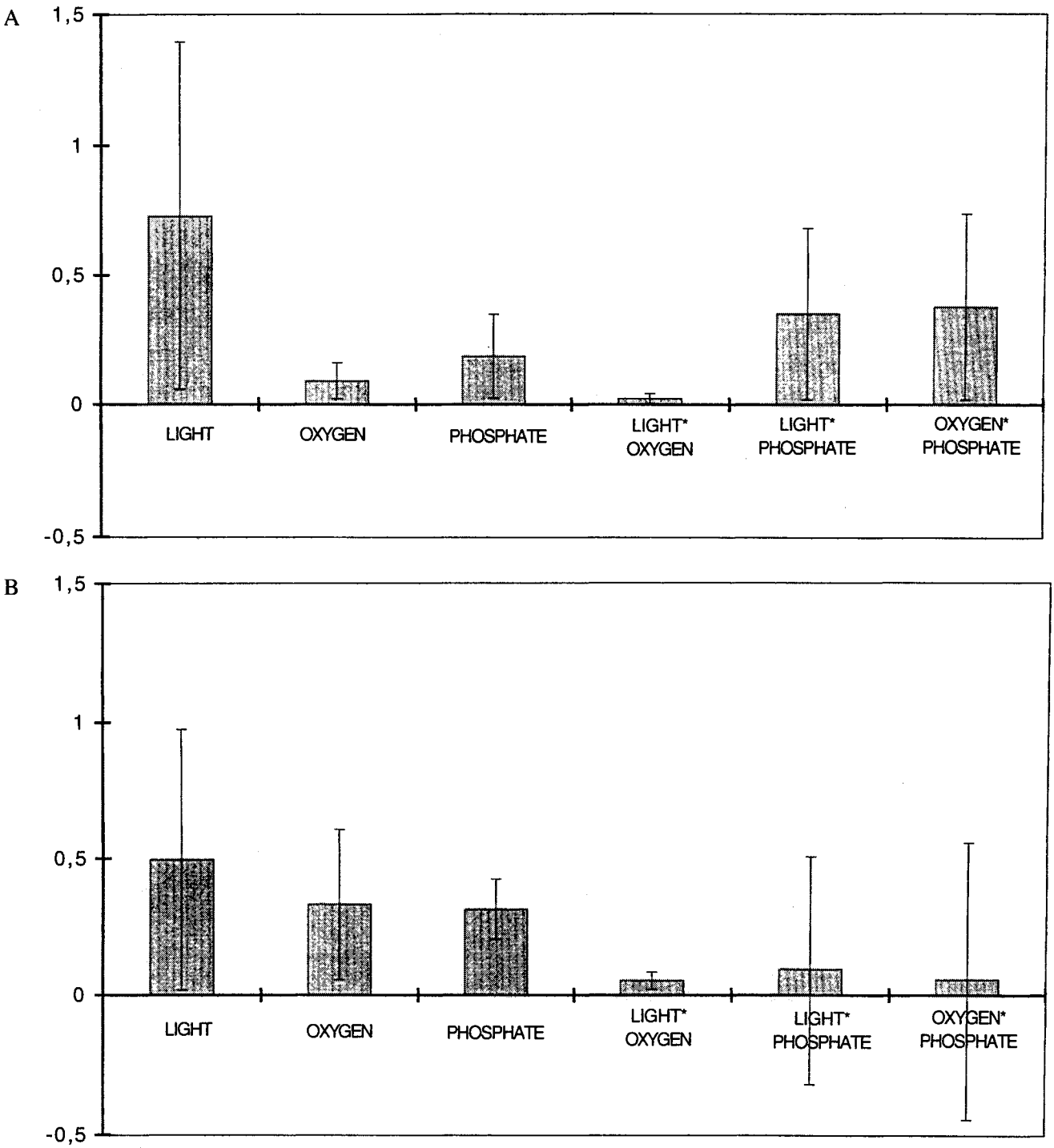

Fig. 2. Scaled and centered PLS coefficients for the oxidation of the methionine residue in hIGF-I at 25°C after 2 (A) and 56 days (B) respectively. The heights of the columns correspond to the change in response estimated for a relative increase of the individual descriptor variable (i.e., from low to high level). The bars indicate confidence level at 90%.

would increase. The pH in the solution with 100 mM phosphate was not affected.

Phosphate-Sulfur Intermediate

Phosphate and methionine can form a phosphorylated sulfonium intermediate (5), which decomposes to methionine sulfoxide and $H_2PO_4^-$, see scheme 2.

$$RS^+R' + HPO_4^{-2} \Leftrightarrow [RR'S^+\text{-}O\text{-}PO_2OH]$$

$$\xrightarrow{OH^-} RSOR' + H_2PO_4^- \quad \text{Scheme 2}$$

The mechanism is also supported by the found pH decrease, as HPO_4^{-2} would be consumed.

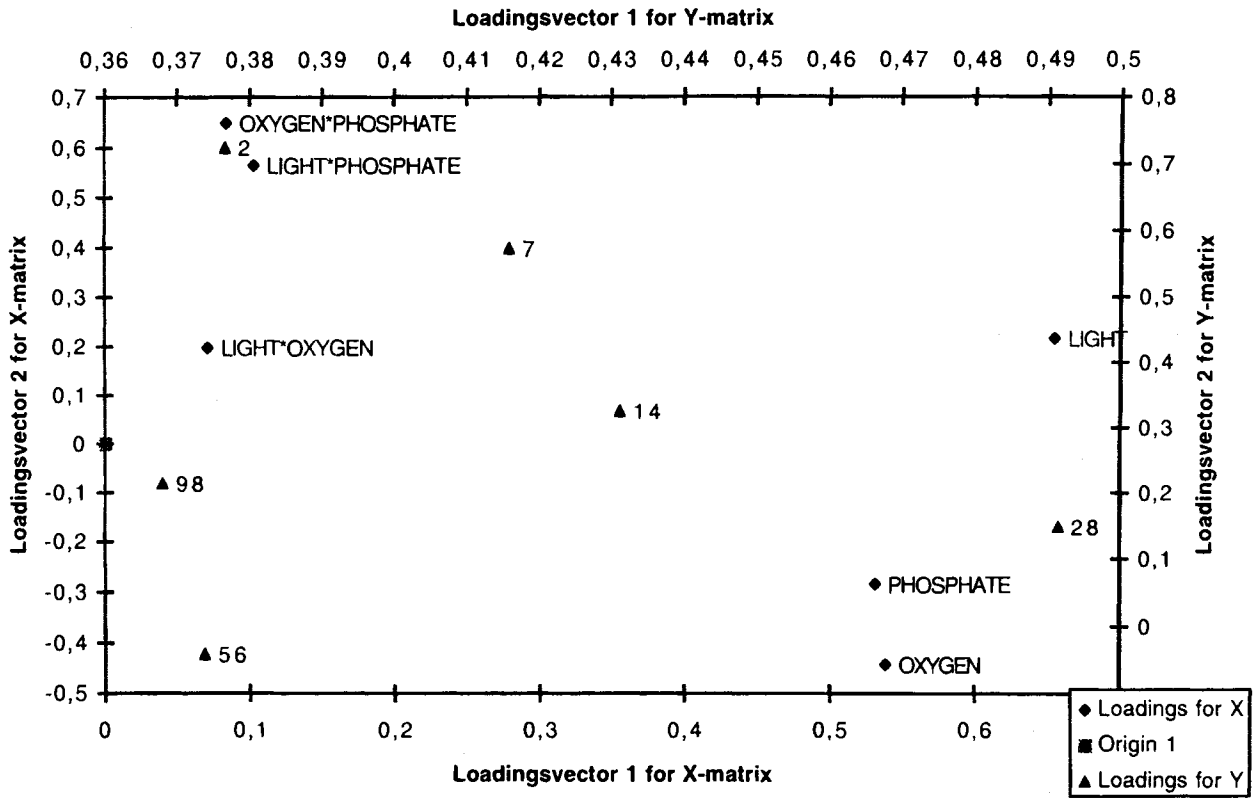

Fig. 3. PLS loading plot showing the first two vectors of PLS loadings. Variables having significant influence have their projected points at the outer edges of the plot. Insignificant variables have their projected point close to the origo in the plot.

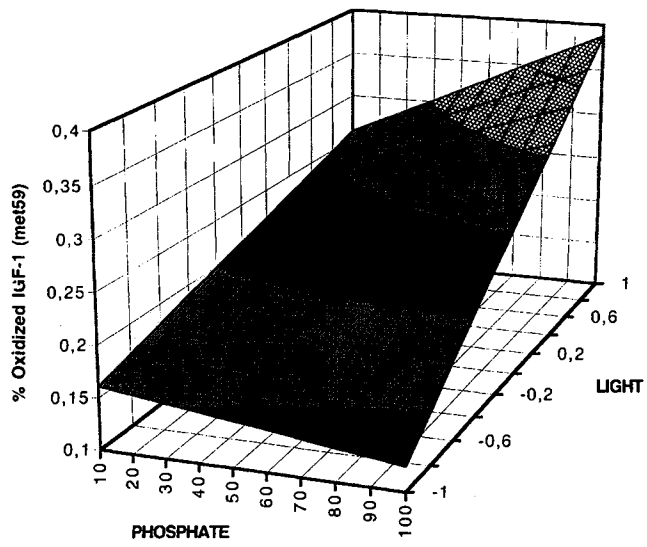

Fig. 4. Response surface for the effects of sodium phosphate in the range 10–100 mM and visible light at a low content of dissolved oxygen (O_2 less than 25 mM) on the oxidation of the methionine residue in hIGF-I at 25°C in aqueous solution. The concentration of hIGF-I was 0.13 mM. ▒, 0. 35–0. 4; ▒, 0. 3–0. 35; ■, 0. 25–0. 3; ■, 0. 2–0. 25; ■, 0. 15–0. 2; ■, 0. 1–0. 15.

Phosphate Radical

Phosphate can be radicalized under photolytic conditions (17). The radical may extract one electron from the nucleophilic sulfur of methionine. This would generate a sulfur radical which could react with e.g. oxygen to form a sulfoxide. Initially, the interaction effect between phosphate and light was significant, see Figure 2, indicating formation of phosphate radicals. This effect decreased with time and was unsignificant at 56 days of storage and thus the phosphate radical cannot explain the positive effect on the oxidation of phosphate.

From the experimental data, the oxidation pathway involving the phosphate-sulfur intermediate appears to be relevant in this study. However, participation of phosphate radicals cannot be ruled out.

Dependence of Oxidation of Methionine upon pH in the Presence of Phosphate

In order to distinguish between the proton donor mechanism and the phosphorylated sulfonium intermediate mechanism of methionine oxidation, the effect of varying the pH was studied. Solutions of 0.13 mM hIGF-I and 50 mM sodium phosphate with pH 6.0, 6.5, 7.0 and 7.5 respectively were prepared and the oxidized methionine was assayed during 3 months. The second order rate constants for the oxidation at each pH value were calculated as previously described (1). As shown in figure 5 a clear dependence of pH for the oxidation

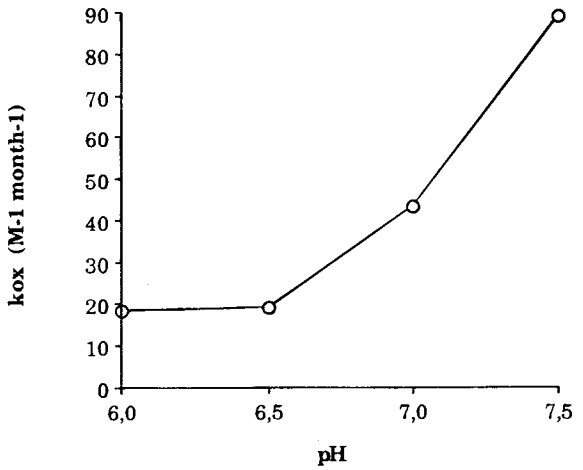

Fig. 5. Second order rate constants for oxidation of methionine in hIGF-I in aqueous solutions with different pH values at 30°C. The solutions were protected from light. The solutions contained 0.13 mM hIGF-I and 50 mM sodium phosphate and had free access to oxygen.

of methionine was found. This supports the phosphorylated sulfonium intermediate mechanism, since HPO_4^{-2} dominates over $H_2PO_4^-$ at higher pH's. Interestingly, these findings are contrary to the previously found decrease in oxidation rates of methionine in small model peptides by dithiothreitol/ferric chloride with increasing pH from 6.0 to 8.0 (18).

CONCLUSIONS

Visible light has a significant effect on the oxidation of methionine in hIGF-I. The oxidation of methionine in hIGF-I appears to follow different pathways in light or in darkness. The absence of any significant interaction between oxygen and light on the oxidation rates indicates that oxygen radicals are not formed during photolysis at these wavelengths. The experimental results rather suggest that a sulfur radical is formed which reacts with molecular oxygen or phosphate. The likely mechanism for the effect of phosphate on methionine oxidation appears to be by pH-dependent formation of a phosphorylated sulfonium intermediate formed from sulfur radicals and phosphate. A significant interaction between light and phosphate also stresses that phosphate stabilizes the suggested sulfur radical. However, the participation of a phosphate radical cannot be ruled out.

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