Comparison between Light Induced and Chemically Induced Oxidation of rhVEGF

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Purpose. The primary objective of this study was to compare the effects of light-and chemical-induced oxidation of recombinant human vascular endothelial growth factor (rhVEGF) and the impact of these reactions on protein formulation.

Methods. A liquid formulation of rhVEGF was exposed to fluorescent light (2×10^4 lux for up to 4 weeks), hydrogen peroxide (H_2O_2), or t-butylhydroperoxide (t-BHP) to induce oxidation of rhVEGF. All samples were then treated by tryptic digest and analyzed by reversed phase HPLC to determine the extent of oxidation. Chemically treated samples were also examined by near-UV and far-UV circular dichroism spectroscopy to determine the effect of oxidation on the structure of the protein.

Results. Exposure to light for 2 weeks resulted in 8 to 40% oxidation of all 6 methionine residues of rhVEGF (Met3 > Met18 > Met55 > Met78,81 > Met94). This amount of oxidation did not affect the binding activity of rhVEGF to its kinase domain receptor (KDR). Light exposure for 4 weeks increased metsulfoxide formation at Met3 and Met18 by an additional 16%, but did not affect the other residues. This oxidation decreased the receptor binding capacity to 73%, possibly due to the role of Met18 in receptor binding. Chemical oxidation of rhVEGF resulted in a greater extent of oxidation at all 6 methionines. Complete oxidation of Met3, Met18 and Met55 was observed after treatment with H_2O_2, while these residues underwent 40 to 60% oxidation after treatment with t-BHP. The receptor binding capacity was significantly reduced to 25% and 55% after treatment with H_2O_2 and t-BHP, respectively. After chemical oxidation, no changes in the secondary or tertiary structure were observed by far-UV and near-UV CD spectroscopy, respectively.

Conclusions. Methionine residues with exposed surface areas greater than 65 Å^2 and sulfur surface areas greater than 16 Å^2 were most susceptible to oxidation. Chemical oxidation resulted in higher metsulfoxide formation and decreased binding activity of the protein to KDR than light-induced oxidation. The reduction in KDR binding was not caused by measurable conformational changes in the protein. Photooxidation was dependent on the amount of energy imparted to the protein, while the ability of t-BHP or H_2O_2 to react with methionine was governed by solvent accessibility of the methionine residues and steric limitations of the oxidizing agent. Significant chemical oxidation occurred on sulfurs with minimum surface areas of 16 Å^2, while increased photooxidation occurred as a function of increasing surface areas of solvent exposed sulfur atoms. Such differences in the extent of oxidation should be considered during protein formulation since it may help predict potential oxidation problems.

KEY WORDS: rhVEGF; oxidation; light; t-butyl hydroperoxide; hydrogen peroxide; methionine sulfoxide.

INTRODUCTION

Monitoring the stability of formulated proteins often includes induction of stress (i.e., exposure to light, agitation, and extreme temperatures) to determine if the integrity of the protein is compromised (1). One of the most common pathways of protein degradation is oxidation of amino acids (Met, Tyr, Trp, Cys, and His). This oxidation can occur through photolytic or chemical reactions, and is dependent on such factors as the temperature, pH, the presence of certain excipients, heavy metals and the presence of molecular oxygen (2,3). Forced oxidation of proteins is commonly done by the addition of oxidants such as hydrogen peroxide, periodate, chloramine-T and t-butyl hydroperoxide (3–5). Here, we will focus on the oxidation of methionine to form metsulfoxide.

Recombinant human vascular endothelial growth factor (rhVEGF) is expressed in *E. coli* and purified as a covalent homodimer. Each monomer is composed of 165 amino acids in two domains (receptor (1-110) and heparin (111-165) binding) and the overall molecular weight of the protein is ~38 kDa. There are 7 intramolecular disulfide bonds per monomer and 2 intermolecular disulfide bonds linking the 2 monomers. Each monomer contains 6 methionines located at residues Met3, Met18, Met55, Met78, Met81, and Met94 in the receptor binding domain (residues 1-110). This domain binds to the cell surface receptors KDR (kinase domain receptor) and Flt-1 (fms-like tyrosine kinase) of endothelial cells to elicit an angiogenic response (6,7). Although VEGF has a higher binding affinity to Flt-1, binding to KDR causes a larger angiogenic response. The receptor-binding epitope of VEGF to KDR and Flt-1 has been identified by charge reversal and alanine scanning mutagenesis (8). The binding of VEGF to KDR is primarily contributed by 5 residues (Phe17, Ile46, Glu64, Gln79, and Ile83) which are grouped into two different areas extending across the VEGF dimer interface. Ile43, Arg82, Pro84, Lys85, and His86 have also been implicated in the binding of VEGF to the KDR receptor (9,10). The positions of Met18, Met78 and Met81 are proximal to the region responsible for binding to the KDR receptor and, thus, the formation of metsulfoxide could have an affect on binding due to the change in polarity. As previously described, rhVEGF is a potent mitogen for vascular endothelial cells, promotes angiogenesis, and increases vascular permeability and vasodilation (11). Whereas rhVEGF may be useful for induction of neovascularization in ischemic disease states, it is also implicated in pathological disorders such as age-related macular degeneration and tumor angiogenesis (12).

In this initial study, an aqueous formulation of rhVEGF was subjected to fluorescent light or treated with t-butyl hydroperoxide (t-BHP) or hydrogen peroxide (H_2O_2). Under acidic conditions, the modification of methionine to metsulfoxide by chemical treatment is the predominant reaction (5). Only metsulfoxide formation has been detected in our studies and further oxidation to the metsulfone derivative and oxi-

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dation of other potential residues have not been observed. Binding of rhVEGF to KDR, but not the Flt-1 receptor, was studied. Light-induced, methionine oxidation of rhVEGF was compared to chemical oxidation. Additionally, the amount of methionine oxidation was compared to the exposed surface areas of the methionine residues and associated sulfur molecules to determine if there was a correlation between oxidation and surface exposure. The apriori assessment of methionine surface exposure may provide early insight into potential stability concerns for protein formulations.

MATERIALS AND METHODS

Materials

The liquid formulation of rhVEGF (formulated with trehalose, Polysorbate 20 and succinate, pH 5.0) was processed and filled at Genentech, Inc. Reagents used for tryptic digest and reversed phase HPLC analysis were analytical grade materials. t-BHP was obtained from Sigma Chemical Co. and H_2O_2 was purchased from Mallinckrodt. TCPK-treated trypsin was purchased from Worthington Biochemical Corporation.

Preparation of Oxidized rhVEGF

For light studies, 3 cc glass vials containing a 1 mL solution of rhVEGF were placed in a ~27°C light box and subjected to fluorescent light at 2×10^4 lux for up to 4 weeks. Vials wrapped in foil were also stored next to the samples as a negative control to light. In addition, vials stored in a cold room at 5°C served as experimental controls. Samples were removed from the light box at 2 weeks and 4 weeks for analysis.

For chemical treatment of the protein, a 1 mL solution of rhVEGF contained in a 3 cc glass vial was treated with either t-BHP or H_2O_2 such that the final concentration of oxidizing agent was 14.6 mM and the molar ratio of oxidizing agent to methionine was approximately 9:1. This concentration of oxidizing agent was chosen based on previous studies performed with t-BHP and rhVEGF (data not shown). A vial of untreated rhVEGF served as a control. All samples were shielded from light and incubated at ambient temperature for 20 h before analysis.

Tryptic Digest

Oxidized rhVEGF samples and controls were prepared for trypsin digestion by diluting the samples in 360 mM Tris buffer containing 8M urea. The samples were then reduced with 1 M dithiothreitol and alkylated with 1 M iodoacetic acid. The rhVEGF was buffer exchanged into a 100 mM Tris, 2 mM calcium chloride buffer, pH 8.3, using a NAP10 column (Pharmacia Biotech). The protein was digested with trypsin at 37°C for 4 h, where the enzyme to substrate ratio was 1:50 (w/w). The digestion reaction was stopped by the addition of 85% phosphoric acid.

Reversed Phase HPLC

Digested rhVEGF was analyzed using a Phenomenex Jupiter 300 C18 column. The protein was eluted using 50 mM sodium phosphate, pH 3.4 (mobile phase A) and acetonitrile (mobile phase B) with 0 to 50% B in 50 min at 40°C at a flow rate of 1 mL/min. The protein was detected by UV absorbance at 214 nm.

KDR Binding Assay

This assay was employed to determine the binding of the rhVEGF to KDR. A microtiter plate was initially coated with anti-human Fc and then incubated with a chimeric IgG molecule (a human Fc fragment attached to 2 KDR molecules). Unbound rhVEGF is removed by washing the plate. A biotinylated antibody to the heparin binding domain of rhVEGF binds to the KDR-rhVEGF complex, then reacts with streptavidin-HRP. The HRP content was measured with a colorimetric substrate (absorbance at 492 nm) and results were normalized to a reference standard.

Circular Dichroism (CD) Spectroscopy

CD analysis of chemically treated and untreated rhVEGF was performed with an Aviv 60/DS spectrometer. Far UV CD measurements were taken from 250 to 190 nm with a step size of 0.2 nm and an averaging time of 3–5 sec. Near UV CD measurements were taken from 360 to 250 nm with a step size of 0.2 nm and an averaging time of 3 sec. Both measurements were performed with a bandwidth of 1.0 nm. Three spectra were taken and averaged for each sample. Near UV spectra were collected by placing 1 mL of 1 mg/mL rhVEGF into a 1 cm circular sample cell (Hellma). Far UV spectra were obtained with the samples (1 mg/mL rhVEGF) placed in 0.1 cm circular sample cell (Hellma). The spectra of buffer samples were collected in the same manner and subtracted from the final protein spectra. Protein spectra were converted to ellipticity by using a mean residue weighting factor of 116.06 for rhVEGF.

Crystallography of rhVEGF

A construct consisting of residues 8-109 of rhVEGF was expressed, refolded and purified. Triclinic crystals were grown from large drops to obtain a typical size of $600 \times 80 \times 80$ μm within 4 weeks, and the crystals were flash-frozen from the drops. Complete data set to 1.9 Å was collected on beamline F1 (lambda = 0.909 Å) at the Cornell High Energy Synchrotron Source equipped with the Princeton CCD-detector. The data set was 94% complete in the resolution range between 20 and 1.0 Å. The total surface area (Å^2) was calculated utilizing a water probe (1.4 Å) rolling over the surface of the rhVEGF.

RESULTS

Effect of Light-Induced Oxidation

Reversed phase HPLC analysis of the tryptic peptides along with peptide sequencing of individual peaks provided a method for detection of methionine oxidation (Table I and Fig. 1). All 6 methionines in rhVEGF were oxidized after exposure to high intensity fluorescent light (Fig. 2). After 2 weeks of exposure, the majority of oxidation occurred at Met3, Met18 and Met55, which were 41%, 22%, and 21% oxidized, respectively. After rhVEGF was subjected to light for 4 weeks, the amount of oxidation at Met3 and Met18

Table I. Tryptic Peptides Containing Methionyl Residues of rhVEGF

Peptide[a]	Residues	Molecular weight[b]	Sequence
T1	1–16	1660.82	APMAEGGGQNHHEVVK
T2	17–23	958.10	FMDVYQR
			SYCHPIETLVDIFQEYPDE
T3	24–56	4092.66	IEYIFKPSCVPLMR
			CGGCCNDEGLECVPTEE
T4	57–82	3065.35	SNITMQIMR
T5	83–101	2229.54	IKPHQGQHIGEMSFLQHNK

[a] Trypsin I was used to cleave the protein on the c-terminal side of Lys and Arg which were not located next to Pro.
[b] The molecular weight of the peptide fragments were computed using the average atomic masses.

increased by an additional 16%, while the amount of oxidation at the other 4 methionines did not change. The foil wrapped samples and samples stored at 5°C for 4 weeks had only slight oxidation at Met3 (8%) and Met55 (5%) and no other oxidization. In addition, exposure of rhVEGF to fluorescent light for 4 weeks resulted in a decrease in KDR binding (73%) compared to the controls and sample exposed to light for 2 weeks (Table II).

Effect of Chemically Induced Oxidation

In comparison to light-induced oxidation of rhVEGF, treatment of the protein in 14.6 mM t-BHP or H_2O_2 (9:1 oxidant to protein) resulted in greater methionine sulfoxide formation at all 6 methionine residues. Treatment of rhVEGF with t-BHP yielded approximately 60% oxidation at Met3 and Met18, and 40% oxidation at Met55 (Fig. 3). Hydrogen peroxide had a greater oxidizing effect on rhVEGF resulting in complete oxidation of these 3 residues as well as significant metsulfoxide formation at Met78, Met81, and Met94. With either oxidant, further oxidation of metsulfoxide to sulfone was not detected by mass spectrometry. Incubation of rhVEGF with chemical oxidants resulted in a significant decrease in binding to KDR (Table II). Chemical treatment of rhVEGF did not induce measurable conformational changes to the protein (Fig. 4). The secondary and tertiary structures of oxidized rhVEGF were similar to the untreated control.

Determination of Surface Areas of Methionine and Sulfur

The exposed surface area ($Å^2$) of a methionine residues from crystallographic data was calculated based on a totally accessible surface of a completely disordered methionine (9). The average exposed surface area for each methionine residue was ranked as follows : Met3 > Met18 >> Met55 > Met81 > Met94 > Met78 (Table III). In addition, the average exposed surface area for the sulfur molecule of each methionine followed the same trend as that observed with the methionines.

DISCUSSION

The purpose of this study was to compare the effects of light and oxidizing agents on metsulfoxide formation of rhVEGF. Without regard to the way in which oxidation was induced, methionine residues with the largest, exposed surface areas (Met3, Met18, and Met55) were most susceptible to oxidation. Those methionines with little or no significant exposed surface area (Met78, Met81, and Met94) were less prone to oxidation because they are folded within the hydrophobic regions of the molecule. Similarly, other proteins such as recombinant human growth hormone (1,13), recombinant human relaxin (14) and recombinant interferon-gamma (4), the exposure of the methionine residue dictated the reactivity to oxidation. In general, a relationship between exposed surface areas of sulfur molecules on the methionines and the extent of oxidation may allow prediction of the probability of protein oxidation.

Light Induced Oxidation

Under light-induced oxidation, methionyl residues with an exposed surface area less than 67 $Å^2$ and sulfur surface areas less than 16 $Å^2$ did not undergo further photooxidation. This oxidation could have occurred by several mechanisms. As discussed by Nema *et al.* (15), photolytic degradation can occur when the drug molecule absorbs the energy emitted from the fluorescent light and then dissipates it in the form of thermal energy, thus creating higher, local temperatures for oxidation of rhVEGF to occur. Photolytic degradation of other proteins have been observed in the presence of nonionic surfactants such as polysorbate 20 which is used at 0.07 mM in the rhVEGF formulation (16,17). At this low concentration, the surfactant is an unlikely mediator as the single source for light-induced oxidation. However, the presence of polysorbate 20 in the rhVEGF liquid formulation could have had an additive effect with light to induce oxidation. Polysorbate 20 has been known to contain low amounts of peroxides, which can, therefore, contribute to metsulfoxide formation and it could act as a photosensitizer (15,16). The energy absorbed by the surfactant can be transferred to rhVEGF, or imparted to react with molecular oxygen to form singlet O_2 ($1\Delta_g O_2$), which is commonly involved in the mechanism for metsulfoxide formation (2) as observed with other proteins (18). Lastly, the photolysis of water can induce the formation of the hydroxyl radical $(HO)\cdot$ (19). Although it is unlikely that oxidation occurs by this pathway, it must be considered as a possible mechanism.

The exposure of rhVEGF to high intensity fluorescent light affected the binding activity of the protein to KDR. Of all the methionines contained in rhVEGF, Met3 has the largest solvent exposed surface area but it is located in a disordered region of the protein that is not involved in KDR binding (8). Because Met55, Met78, Met81, and Met94 appear to have reached maximum levels of light-induced oxidation and

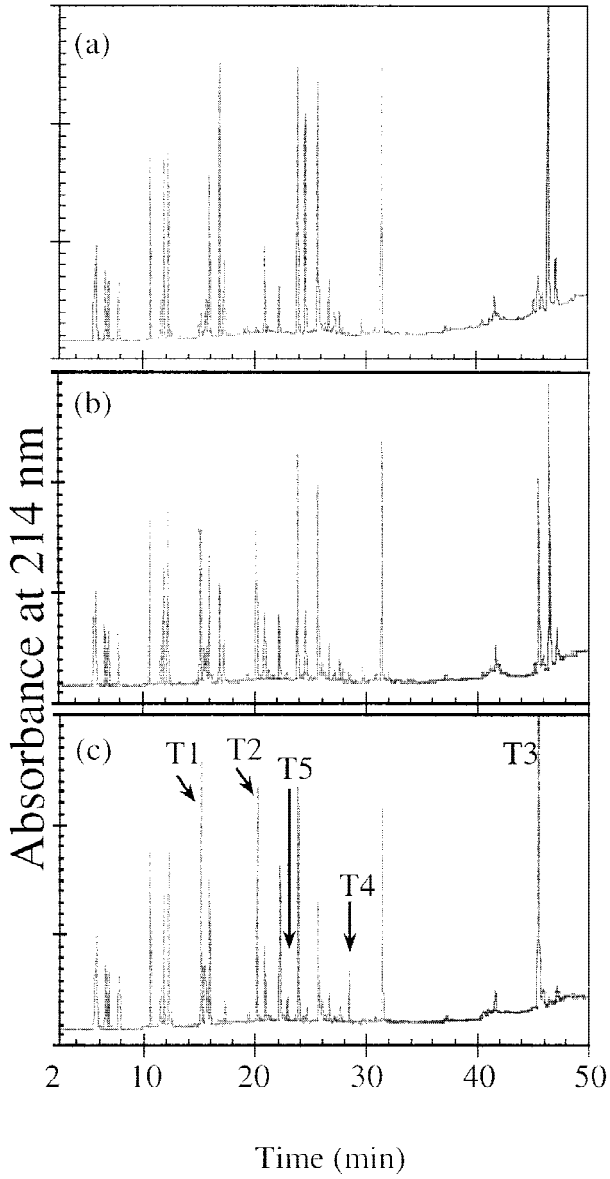

Fig. 1. RP-HPLC tryptic digests of rhVEGF before and after treatment with t-BHP or H_2O_2. Chromatograms from top to bottom are samples of (a) untreated rhVEGF, (b) rhVEGF treated with 14.6 mM t-BHP, and (c) rhVEGF treated with 14.6 mM H_2O_2. T1, T2, and T3 represent the oxidized form of Met3, Met18, and Met55, respectively. T4 represents the oxidized form of Met78 and Met81, and T5 represents Met94. Methionine residues were not present in residues 102-165 and this region contained several lysine and arginine residues, yielding several small, undetectable peptides after tryptic digest. Similar chromatograms were observed for light-induced oxidation.

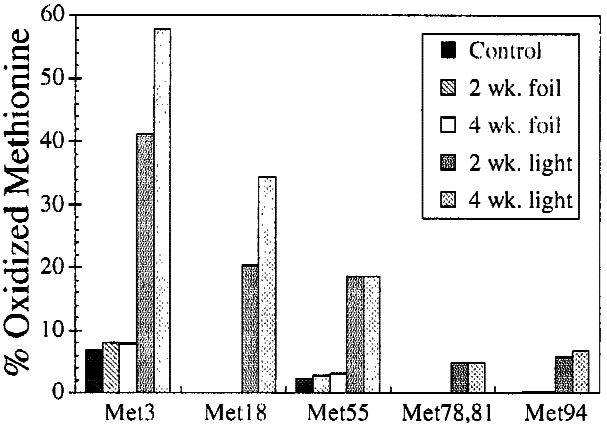

Fig. 2. The percent of oxidized methionine for rhVEGF after tryptic digest of light-treated samples. Foiled and unfoiled vials containing a solution of rhVEGF were stored at ~27°C and subjected to fluorescent light at 2×10^4 lux for up to 4 weeks. An unfoiled vial stored at 2-8°C also served as a control.

are proximal to the KDR binding domain, it is predicted that the change in polarity due to oxidation of Met18 has a significant effect on the binding capacity.

Chemically Induced Oxidation

The treatment of rhVEGF with chemical oxidants resulted in significant oxidation at all 6 methionines. The treatment of the protein with 14.6 mM H_2O_2 completely oxidized those residues with surface areas greater than 65 $Å^2$ (Met3, Met18, and Met55), and significant oxidation of the other 3 methionines with less exposed surfaces. Even though the mechanism of oxidation observed for both t-BHP and H_2O_2 was nucleophilic substitution (20), the amount of oxidation was significantly different between the two oxidizing agents. With nucleophilic substitution, a succession of electronic displacements occurs as the electrons of the hydroxyl group of t-BHP or H_2O_2 react with the electrons on the sulfur molecule of methionine. The physical size of the oxidant could then determine the extent of oxidation, and the accessibility of the hydroxyl group to the sulfur may be different between the two oxidants. The 3 methyl groups of t-BHP may sterically hinder the chemical reaction or prevent access to the sulfur. Hydrogen peroxide, however, is smaller and therefore has greater ability to diffuse into the protein surface and react with methionine. It has also been reported by Keck that t-BHP is a methionine-specific oxidant to exposed methionyl residues (4), while H_2O_2 can elicit non-specific oxidation (21) and cannot differentiate between exposed and buried methionines, as evidenced by oxidation of Met78, 81, and Met94, which are all buried methionines. The exposure of rhVEGF to these oxidizing agents also had a significant effect on binding to KDR. Treatment of rhVEGF with H_2O_2 resulted in a greater loss of receptor binding activity due to a change in polarity of Met18 as well as Met81, both of which are located

Table II. Binding Activity of Treated and Untreated rhVEGF to KDR

Treatment	Storage	% Activity[a] ± 1 SD
Light	2 wks.	110 ± 12
Light	4 wks.	73 ± 15
Foil	2 wks.	100 ± 20
Foil	4 wks.	100 ± 19
14.6 mM H_2O_2	20 hrs.	25 ± 2
14.6 mM t-BHP	20 hrs.	55 ± 3
Untreated	none	100 ± 7

[a] %Activity is defined by the amount of rhVEGF binding to KDR relative to a reference standard of known concentration and established at 100% activity.

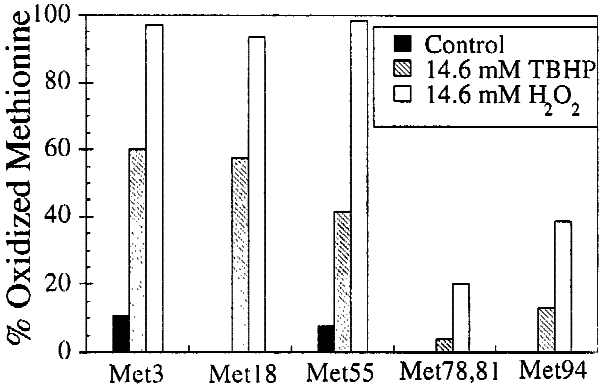

Fig. 3. The percent of oxidized methionine for rhVEGF after tryptic digest of chemically-treated samples. Foiled vials containing rhVEGF in 14.6 mM t-BHP or H_2O_2 were stored at room temperature for 20 h. A foiled vial containing untreated rhVEGF was also stored at room temperature and served as a control.

near the binding site. Hydrogen peroxide has the ability to oxidize tyrosines located at residues 21 and 45, which are proximal to the binding interface of rhVEGF, but these experiments were performed at low pH where oxidation of tyrosines is unlikely. Furthermore, no evidence of oxidation of other residues was observed by tryptic mapping. The loss in KDR binding of chemically treated rhVEGF was not due to measurable conformational changes in the protein based upon far-UV and near-UV CD spectra. This observation does not, however, eliminate the possibility that a small, local, structural perturbation occurred upon oxidation, resulting in a decreased affinity of rhVEGF for KDR.

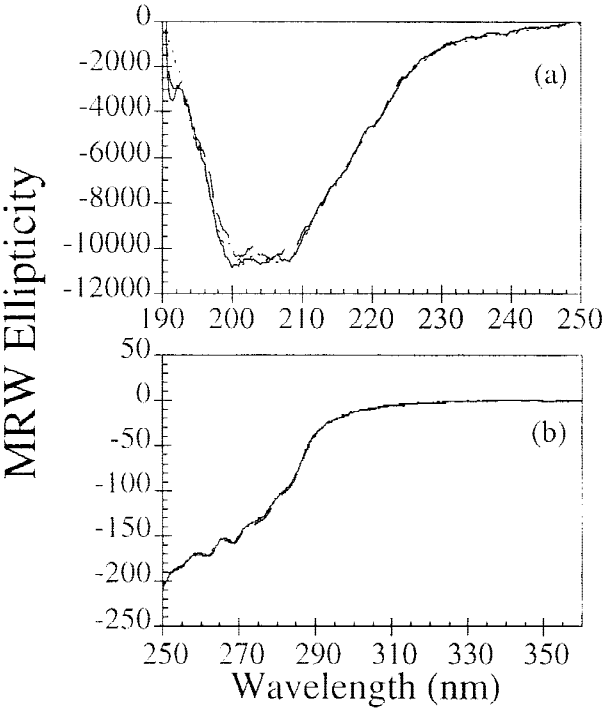

Fig. 4. CD spectra of rhVEGF before and after treatment with t-BHP or H_2O_2. Results from top to bottom are spectra scanned at (a) far-UV, and (b) near UV for untreated rhVEGF (——), and samples treated with 14.6 mM t-BHP (- - -) or 14 mM H_2O_2 (– –).

Table III. Exposed Surface Areas of Methionine and Sulfur for rhVEGF

	Average surface area[a], Å^2	
Met residue	Methionine	Sulfur
3	200.0	46.0
18	109.8	30.5
55	67.3	16.3
78	0.3	0.0
81	36.0	8.2
94	7.3	0.0

[a] The average surface area was calculated as the totally accessible surface for a completely disordered methionine. The sulfur molecules from methionine residues 78 and 94 were determined to have nearly nonexistent exposed surface areas.

Comparison between Light Induced Oxidation and Chemically Induced Oxidation

To determine if the same route of oxidation had occurred between samples exposed to high intensity light and those exposed to oxidizing agents, the amount of metsulfoxide formation was plotted as a function of the exposed surface area of the sulfurs for each methionine (Fig. 5). Without regard to the method used to induce oxidation, Met3, Met18, and Met55 showed the greatest amount of methionine sulfoxide formation since they are the methionines with the greatest exposed surface areas. Chemical oxidation occurred more rapidly than photolytic degradation. Treatment of rhVEGF with the oxidizing agents resulted in maximum oxidation of methionines exhibiting minimum sulfur surface areas of 16 Å^2 and 30 Å^2 for H_2O_2 and t-BHP, respectively. Increased photooxidation occurred in a time dependent manner as a function of the sulfur surface and those sulfurs with surface areas less than 16 Å^2 did not undergo further oxidation. A similar relationship between metsulfoxide formation and exposed surface areas was also observed in the oxidation of recombinant human relaxin by H_2O_2. Relaxin contains 2 methionines located at residues 4 and 25, and the exposed surface area for each sulfur atom is 9 and 15 Å^2, respectively (22). It has been demonstrated by Nguyen *et al.* that the rate of oxidation at Met25 is 2.5 times faster than that observed at Met4 (14).

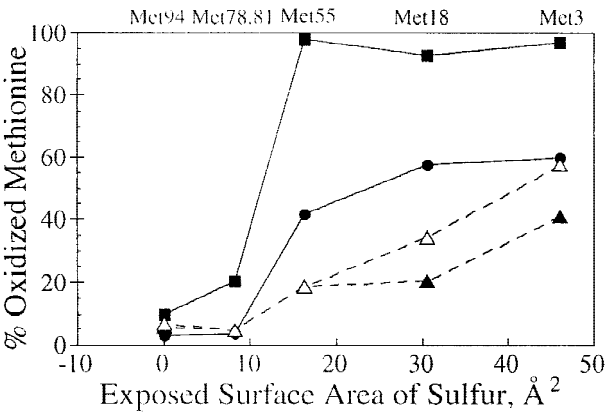

Fig. 5. The percent metsulfoxide formation of rhVEGF from samples stored in 14.6 mM H_2O_2 (■) or 14 mM t-BHP (●), or from samples exposed to fluorescent light for 2 weeks (▲) and 4 weeks (△) were plotted as a function of exposed surface area of the sulfur molecule.

Under the conditions used to induce photooxidation of rhVEGF, the majority of the thermal energy that is emitted by the light source would be initially absorbed onto the surface of the entire protein; whereas with chemical oxidation, there is greater opportunity for all methionines to undergo oxidation. The amount of energy from light that is absorbed specifically by the methionines depends on the exposure of those residues. Those methionines with large, exposed surface areas would immediately absorb this energy while buried methionyl residues would require greater energy penetration of the protein to achieve oxidation. On the other hand, depending on the oxidant used, the chemical reacts only with specific residues. Although the extent of oxidation induced by H_2O_2 and t-BHP was different (see above), treatment with chemical oxidizers provides a greater potential for oxidation than the thermal energy imparted by fluorescent light. These findings suggest that photooxidation follows a different mechanism than that observed with chemical oxidation. Overall, determination of the exposed surface areas of methionyl residues and their sulfurs may help scientists to design formulations that minimize oxidation of proteins with potentially reactive methionine residues.

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