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HYDROGEN PEROXIDE MODIFICATION OF HUMAN OXYHEMOGLOBIN

ROBIN P. STEFFEK[§] and MICHAEL J. THOMAS

Department of Biochemistry, Wake Forest University Medical Center, The Bowman Gray School of Medicine, Winston-Salem, North Carolina 27103, U.S.A.

The effect of H_2O_2 on the primary structure of OxyHb was studied. Upon treatment of OxyHb with H_2O_2 ([Heme]/[H_2O_2] = 1), tryptophan and methionine residues of the β-chain were modified. Treatment of ApoHb with H_2O_2 resulted in the modification of histidine and methionine residues in both globin chains. Tryptophan residues were unaffected. Modification of methionine residues in both the β-chain of OxyHb and ApoHb probably results from the direct oxidation of methionine by H_2O_2. The modification of histidine residues in ApoHb may be mediated by a metal-catalyzed oxidation system comprised of H_2O_2 and histidine-bound iron. The H_2O_2-mediated modification of tryptophan in the OxyHb β-chain, however, requires the heme moiety.

KEY WORDS: Oxyhemoglobin, apohemoglobin, hydrogen peroxide, amino acid modification.

ABBREVIATIONS: OxyHb, human oxyhemoglobin A; Hb, human hemoglobin A; heme, iron protoporphyrin IX regardless of oxidation state; ApoHb, hemoglobin from which the heme has been removed; O_2^-, superoxide anion radical; ·OH, hydroxyl radical; EDTA, ethylenediaminetetraacetic acid; RBC's, red blood cells; RP-HPLC, reversed phase high performance liquid chromatography; TFA, trifluoroacetic acid; ACN, acetonitrile; Met, methionine; Trp, tryptophan; His, histidine; Tyr, tyrosine.

INTRODUCTION

Hemoglobin A (Hb), comprising ~66% of the total iron within an adult human, has been characterized as a biologically hazardous "Fenton"reagent due to its ability to accelerate the peroxidation of arachidonic acid and lipids within the red blood cell (RBC) membrane.[1] However, the exact identity of the oxidizing species responsible for promoting lipid peroxidation in the presence of Hb has been a subject of much dispute. Recently, Puppo and Halliwell[2] have conclusively demonstrated that ·OH is generated in the reaction of OxyHb with a large molar excess of H_2O_2. Interestingly, the oxyradical species formed from the reaction of OxyHb with H_2O_2 is influenced by the [Heme]/[H_2O_2] ratio. This study also provided evidence for the formation of an oxidizing species at a [Heme]/[H_2O_2] ratio of 1:1 which is much less reactive than ·OH.

Free OxyHb, acting as a catalyst in the formation of ·OH, could promote the destruction of a variety of biomolecules. Numerous literature accounts indicate that active oxygen species (eg. ·OH and O_2^-) and H_2O_2/Fe or Cu systems alter protein structure by initiating amino acid modifications, protein fragmentation and crosslinking.[3-6] Thus, in addition to its role as a catalyst, the Hb molecule is itself undoubtedly

[§]To whom correspondence should be addressed.

a primary target of oxyradical-induced modification. Isoelectric focusing studies[7] have in fact shown that the protein portion of Hb is sensitive to oxidative modification by high fluxes of $\cdot OH$ or $\cdot OH + O_2^-$.

The chemical effects of H_2O_2 on the primary structure of OxyHb have not been extensively studied. A study of the H_2O_2-mediated modification of OxyHb could not only provide information pertinent to the nature of the oxidant formed from the reaction of OxyHb with H_2O_2 at a 1:1 [Heme]/[H_2O_2] ratio, but also insights into the mechanism(s) by which Hb catalyzes the oxidative modification of biologically important molecules.

We have begun to study the H_2O_2-induced modification of OxyHb at a [Heme]/[H_2O_2] ratio of 1:1. The aim of this research is to determine the extent and specificity of protein modification at the amino acid level under these conditions. The role of heme iron in mediating Hb modification is also investigated.

MATERIALS AND METHODS

All inorganic reagents were of the highest quality available from J.T. Baker, unless stated otherwise. All solutions were prepared with Milli-Q water (Millipore). Amino acid analysis was performed using the Waters "Pico-Tag" method.

Preparation of Oxyhemoglobin A

OxyHb was isolated from freshly drawn blood collected with EDTA (100 mg/100ml blood; Sigma) as the anticoagulent. RBC's were washed three times with an equal volume of 0.9% NaCl. Hemolysis of the cells was achieved by addition of an equal volume of Milli-Q water. Cell debri was removed by centrifugation at 30,000 g for 35 min at 4°C. OxyHb was freed from other red cell proteins (eg. catalase and superoxide dismutase) by column chromatography on DEAE-Sephadex according to the method of Huisman and Dozy.[8] The major peak of OxyHb from the column was concentrated and exchanged into KOH/KH_2PO_4 buffer using Centricon ultrafiltration microconcentrators (Amicon) before use. OxyHb concentration was determined from the A_{577nm} by using $\varepsilon_{577nm} = 15{,}000\ M^{-1}cm^{-1}$ (expressed per heme group).[9]

Oxidation of OxyHb by H_2O_2

OxyHb (58 μM heme) was incubated with H_2O_2 (58 μM) at 37°C in 25 mM KOH/KH_2PO_4 buffer (pH 7.4) for 5 and 60 min. Control samples were incubated under the same conditions in the absence of H_2O_2. The total reaction volume was 1 ml. Reactions were terminated by dilution with Milli-Q water and ultrafiltration at 4°C using Centricon microconcentrators. H_2O_2 solutions were prepared by dilution of 30% H_2O_2 (Mallinkrodt) with Milli-Q water just prior to use. H_2O_2 concentration was determined from the A_{240nm} using $\varepsilon_{240nm} = 43.6\ M^{-1}cm^{-1}$.[10]

Oxidation of ApoHb by H_2O_2

ApoHb was prepared by the Acid-Acetone method.[11] ApoHb samples (15.6 μM) were incubated with H_2O_2 at [ApoHb]/[H_2O_2] ratios of 1:1 (15.6 μM) and 1:60 (936 μM) at 37°C in 25 mM KOH/KH_2PO_4 buffer (pH 7.4) for 60 min. Control samples were

incubated under the same conditions in the absence of H_2O_2. The total reaction volume was 2 ml. The reactions were terminated by quick-freezing in a dry ice/acetone bath followed by lyophilization.

Proteolytic Digestion

Individual globin chain and ApoHb samples were dissolved in 25 mM NH_4HCO_3 buffer (pH 8) and treated with trypsin (Boehringer Mannheim) for 24 hr at 37°C. Trypsin was added in a quantity equal in weight to 1% of the protein to be hydrolyzed at 0 and 12 hr. The final mixture was quick-frozen and lyophilized.

HPLC of Globin Chains and Peptides

Separation of H_2O_2 treated and control globin chains was accomplished by RP-HPLC on a Vydac C_4 column (The Separations Group, Hesperia, CA) with 80/20 (v/v) H_2O/ACN, 0.1% TFA (A) and 40/60 (v/v) H_2O/ACN, 0.1% TFA (B) as eluents. The gradient for separation ran from 44 to 56% B in 60 min at a flow rate of 1 ml/min.[12] Eluent absorbance was monitored at 220 nm. Separation of the soluble Hb tryptic peptides was performed on a Vydac RP-C_{18} large-pore column with 0.1% TFA (A) and 0.1% TFA in ACN (B) as eluents. The gradient for separation ran from 0 to 56% B in 120 min at a flow rate of 1 ml/min.[13] Eluent absorbance was monitored at both 220 and 280 nm.

RESULTS

Separation of H_2O_2-treated Globin Chains

After reacting OxyHb with H_2O_2, each sample was chromatographed on a Vydac RP-C_4 HPLC column to separate the globin chains. Significant changes in the chromatographic behavior of the globin chains are observed upon treatment of OxyHb with H_2O_2 ([Heme]/[H_2O_2] = 1) for 60 min (Figure 1). The elution profile of H_2O_2-treated OxyHb globin chains shows an increase in the retention times of the α- and β-chains by 2.0 and 3.5 min, respectively (Figure 1B). An alteration of the tertiary structure of the globin chains as a result of amino acid modification would explain such changes. These changes are accompanied by a 40% decrease in the intensity of the 220 nm absorbance of the β-chain, relative to control. In contrast, the relative intensity of the 220 nm absorbance of the α-chain was unaltered. The presence of an additional peak at 43 min may indicate the crosslinking of a small proportion of β-chains, but this phenomenon has yet to be investigated. Similar shifts in retention time were observed in the elution profile of the OxyHb sample incubated under the same conditions for 5 min. The relative intensity of the 220 nm absorbance of the β-chain was reduced by $\sim$ 15% in this case.

Tryptic Peptide Map Analysis of H_2O_2-treated Globin Chains

In order to determine the sites of H_2O_2-induced modification, the individual α- and β-chains of each sample were subjected to tryptic digestion and the resulting peptide fragments chromatographed on a Vydac RP-C_{18} HPLC column. The α-chain soluble tryptic peptides derived from the samples of OxyHb treated with H_2O_2 for 5 and

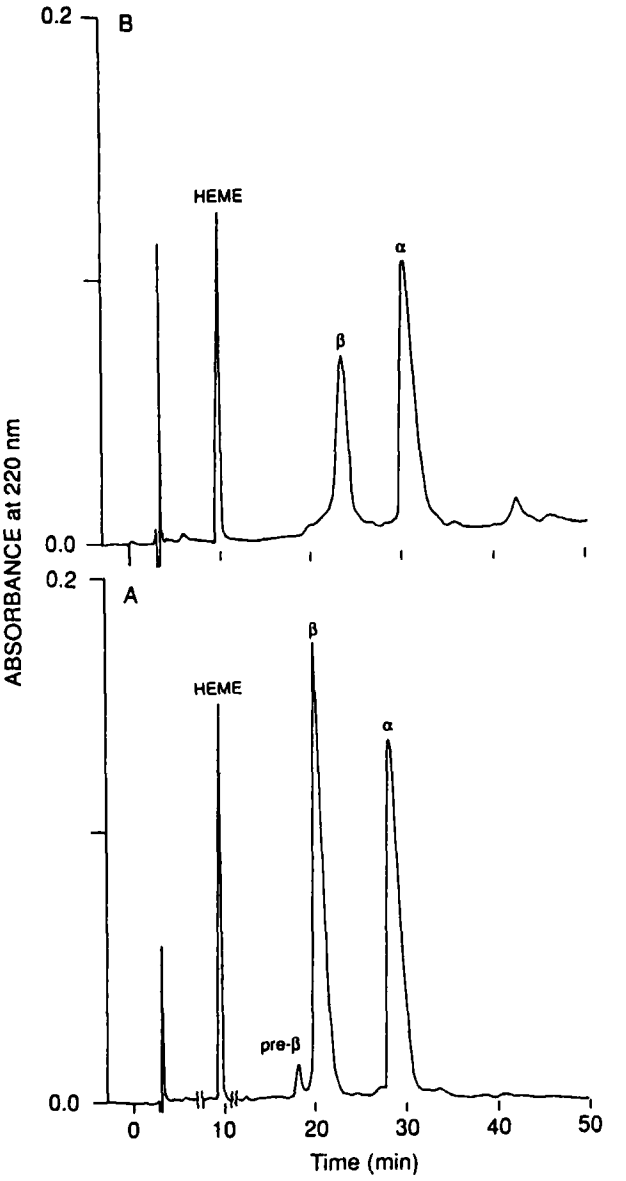

FIGURE 1 RP-HPLC separation of globin chains on a Vydac C_4 column. A) control OxyHb. B) OxyHb treated with H_2O_2 ([Heme]/[H_2O_2] = 1) for 1 hr at 37°C. Refer to "Materials and Methods" for gradient conditions.

60 min were identical to those of the unmodified α-chain (data not shown). In contrast, the tryptic peptide map of the β-chain peptides derived from the OxyHb sample treated with H_2O_2 for 60 min revealed that the relative intensities of the 220 nm and 280 nm absorbances of the native β-chain peptides; βT-2 and βT-4 + βT-5 were reduced (Figure 2B). The 280 nm absorbance is indicative of the total tryptophan (Trp) and/or tyrosine (Tyr) content of the peptide peaks. The βT-2 peptide contains

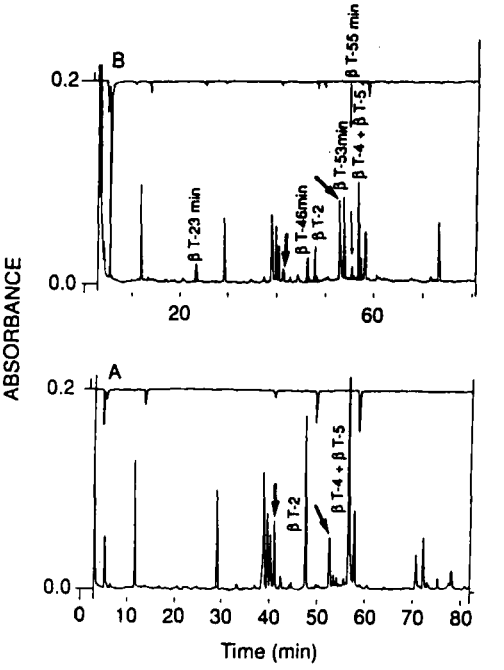

FIGURE 2 RP-HPLC separation of β-chain tryptic peptides from A) control and B) H_2O_2-treated ([Heme]/[H_2O_2] = 1, 1 hr at 37°C) OxyHb on a Vydac C_{18} column. The top and bottom elution traces in each tryptic map represent the absorbance profiles at 280 and 220 nm, respectively. The 280 nm trace is offset 3 min from the 220 nm trace. Gradient conditions described under "Materials and Methods".

a single Trp residue, while the βT-4 peptide contains a Trp and a Tyr residue. The βT-5 peptide, which coelutes with βT-4, does not contain a Trp residue. Approximately 30% of each of the two peptides; βT-2 and βT-4 were oxidatively modified, based upon the observed decrease in the 280 nm absorbances of the native peptides (Figure 2B). In addition, four new peptides; βT-23 min, βT-46 min, βT-53 min and βT-55 min, appear in the β-chain tryptic map (Figure 2). Three of these peptides (βT-23 min, βT-46 min and βT-55 min) have a detectable 280 nm absorbance. Treatment of OxyHb with H_2O_2 for 5 min resulted in only minimal changes to the βT-2 and βT-4 + βT-5 peptides. While the βT-23 min, βT-53 min and βT-55 min peptides were not detected in this sample, a peak having a detectable 280 nm absorbance was observed at an elution time of 46 min.

Amino acid composition analysis of the βT-53 min peptide has been completed. This peptide has the following composition: Asp (2), Glu/Gln (2), Ser (2), Gly (2), Thr (1), Ala (1), Pro (2), Val (1), Met (0.2), Leu (1), Phe (3), Lys (1). The composition of the βT-53 min peptide differs from that of the native βT-5 peptide only in Met content. The native βT-5 peptide contains one Met residue.

The other obvious differences between chromatograms A and B of Figure 2 are those indicated by bold arrows and those past the elution time of 70 min. These differences may not be due to H_2O_2 treatment, but rather to the variability in the yield of the β-chain, cysteine-containing, "core" peptides caused by solubility limitations and incomplete proteolytic cleavage.

Tryptic Peptide Map Analysis of H_2O_2-treated ApoHb

In order to determine whether the heme moiety was required for the observation of the pattern of H_2O_2-induced β-chain modifications, ApoHb samples were treated with H_2O_2 at [ApoHb]/[H_2O_2] ratios of 1:1 and 1:60, lyophilized and digested with trypsin. Trypsin hydrolysates, containing both α-and β-chain peptides, were chromatographed by RP-HPLC. As demonstrated in the tryptic map of ApoHb treated with a [ApoHb]/[H_2O_2] of 1:60 for 60 min (Figure 3B), the αT-7, βT-7, αT-5 and αT-8,9 peptides are conspicuously absent. In addition, a decrease in the relative intensity of the 220 nm absorbance of the βT-4 + βT-5 peak is observed in the absence of any change in the intensity of the corresponding 280 nm absorbance, thus indicating that the βT-5 peptide is partially modified. The decreased intensity of the βT-5 peptide and disappearance of the αT-5 and αT-8,9 peptides coincide with the appearance of four new peptides; T-53 min, T-49 min, T-58 min and T-59 min. The βT-5, αT-5 and αT-8,9 peptides each contain a Met residue. Thus, the new peptides (T-49, T-53, T-58 and T-59 min) presumably arise from the H_2O_2-induced oxidation of Met to the sulfoxide. The last significant change in this peptide map (Figure 3B) is the decrease in both the 220 nm and 280 nm absorbances of the small βT-15 peptide (His-Tyr).

It is important to note that while treatment of OxyHb with H_2O_2 ([Heme]/[H_2O_2] = 1) results in a decrease in the relative intensity of the 280 nm absorbances of the native βT-2 and βT-4 peptides and the appearance of three new peaks each possessing a 280 nm absorbance (Figure 2B), such changes are not observed after

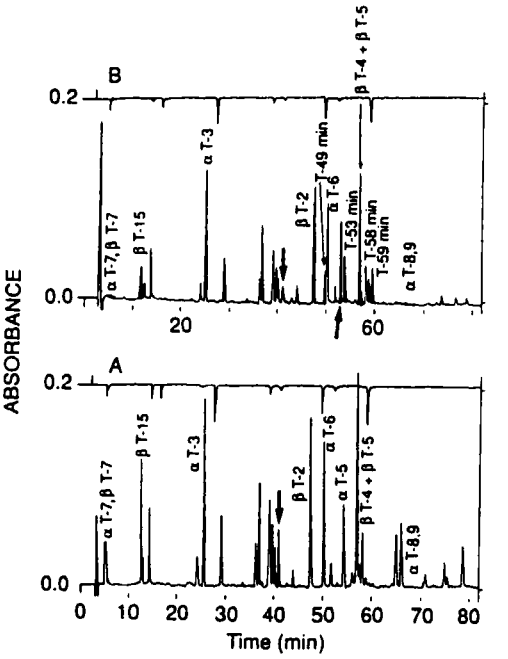

FIGURE 3 RP-HPLC separation of tryptic peptides from A) control and B) H_2O_2-treated ApoHb ([ApoHb]/[H_2O_2] = 1:60, 1 hr at 37°C) on a Vydac C_{18} column. The top and bottom elution traces in each tryptic map represent the absorbance profiles at 280 and 220 nm, respectively. The 280 nm trace is offset 3 min from the 220 nm trace. Bold arrows indicate β-chain "core" peptides. Gradient conditions described under "Materials and Methods".

treatment of the ApoHb with H_2O_2 ([ApoHb]/[H_2O_2] = 1:60) (Figure 3B). The tryptic map of the ApoHb sample treated with H_2O_2 at a [ApoHb]/[H_2O_2] ratio of 1:1 for 60 min (data not shown) was similar to that obtained at the higher H_2O_2 concentration, except that the extent of modification to methionine-containing peptides was not as pronounced.

DISCUSSION

The extent of H_2O_2-induced modification to the primary structure of OxyHb was investigated by RP-HPLC. This chromatographic technique is sensitive to slight alterations in amino acid sequence of proteins and peptides. Although the shifts in the elution times of the α- and β- chains would indicate that both chains are significantly altered after exposure to H_2O_2, the α- and β-chain C_{18} tryptic maps show that the readily detectable oxidative modifications are localized to the β-chain. The irreproducible elution of "core" peptides from the RP-C_{18} HPLC column may, however, explain the discrepancy in the results of the RP-C_4 and RP-C_{18} HPLC analyses of the α-chain which contains a single cysteine residue. It is also evident, from a comparison of Figure 2A and Figure 2B, that the H_2O_2-induced modifications within the β-chain are site-specific. Furthermore, the tryptic maps obtained from H_2O_2-treated OxyHb and H_2O_2-treated ApoHb demonstrate that the pattern of oxidative modification sustained by the globin chains is heme-dependent. His and Met residues are modified in the absence of the heme moiety, while Trp residues are modified in the presence of heme.

The H_2O_2-mediated modification of His residues in ApoHb is evident from the absence of the αT-7 (Gly-His-Gly-Lys) and βT-7 (Ala-His-Gly-Lys) peptides of ApoHb (Figure 3B). Histidine has been shown to be susceptible to oxidation by a metal-catalyzed system comprised of Fe^{+2} and H_2O_2.[14] Iron released by the heme moiety in the preparation of ApoHb may be bound by His and undergo a reaction with H_2O_2 to generate an active oxygen species which oxidizes His. Conversion of the His residue within the αT-7 and βT-7 peptides into a residue of increased polarity could result in the formation of tryptic peptides which elute at the solvent front, thus explaining their absence from the tryptic map.

The oxidative modification of Met residues in both OxyHb and ApoHb is presumably due to the direct reaction of H_2O_2 with the thioether functionality. The thioether groups of Met side chains in proteins are known to be susceptible to oxidation by H_2O_2, particularly under acidic conditions.[15,16] The product of Met oxidation is methionine sulfoxide. Met sulfoxide could not be detected in the βT-53 min hydrolysate by the "Pico-Tag" method of amino acid analysis because the 6 M HCl used in this method reduces Met sulfoxide back to Met. Quantitative determination of the βT-5 peptide amino acids, except Met, in the βT-53 min hydrolysate indicates that the βT-53 min peptide is formed as a result of the modification of the Met residue in the βT-5 peptide.

Exposure of OxyHb to H_2O_2 at a [Heme]/[H_2O_2] ratio of 1:1 results in the concommitant decrease in the 280 nm absorbance of two native β-chain Trp containing peptides and the appearance of the βT-23 min, βT-46 min and βT-55 min peptides, thus indicating that these new peptides result from the H_2O_2-mediated modification of Trp^{15} and Trp^{37} which are found in the βT-2 and βT-4 peptides, respectively. The observed 40% decrease in the 220 nm absorbance of the intact H_2O_2-treated β-chain is an additional piece of data which indicates that the Trp residues of the βT-2 and

βT-4 peptides are modified, since the indole moiety makes a substantial contribution to the intensity of the 220 nm absorbance. While it is known that the oxidation of Trp residues within proteins occurs upon exposure to a minimum of 15 equivalents of aqueous alkaline H_2O_2,[17] the C_{18} tryptic map of H_2O_2-treated ApoHb indicates that Trp is not directly oxidized under the experimental conditions used in this study. The findings of Dryhurst *et al.*[18] and Hoffman *et al.*,[19] indicating the susceptibility of the indole functionality to heme-mediated oxidation by H_2O_2, are consistant with our observation of heme-dependent H_2O_2-induced modification of β-chain Trp residues. Current research efforts are focused upon determining the identity of the product of Trp modification in order to provide a clearer understanding of the biochemical mechanisms involved in the modification of OxyHb by H_2O_2.

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