MECHANISM OF HYDROGEN PEROXIDE-INDUCED FORMATION OF DITYROSINE AND TYROSINE OXIDATION PRODUCTS IN HEMOGLOBIN AND RED BLOOD CELLS AND THEIR RELEASE BY PROTEOLYSIS *

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ABSTRACT

Oxyhemoglobin exposed to a continuous flux of H\textsubscript{2}O\textsubscript{2} underwent oxidative modifications, including limited release release of fluorescent fragmentation products. The main fragments formed were identified as oxidation products of tyrosine, including dopamine, dopamine quinone, and dihydroxyindol. Further release of these oxidation products plus dityrosine was only seen after proteolytic degradation of the oxidatively modified hemoprotein. A possible mechanism is proposed to explain the formation of these oxidation products which includes cyclization, decarboxylation and further oxidation of the intermediates. Release of dityrosine is proposed as a useful technique for evaluating selective proteolysis after an oxidative stress, because dityrosine is metabolically stable, and it is only released after enzymatic hydrolysis of the oxidatively modified protein. The measurement can be accomplished by High Performance Liquid Chromatography (HPLC)* with fluorescence detection, or by high efficiency thin layer chromatography. Comparable results, in terms of dityrosine release, were obtained using red blood cells (RBC) of different sources after exposing them to a flux of H\textsubscript{2}O\textsubscript{2}. Furthermore, dityrosine has been reported to occur in a wide variety of oxidatively modified proteins. These observations suggest that dityrosine formation and release can be used as a highly specific marker for protein oxidation and selective proteolysis.

* ABBREVIATIONS: HPLC, High Performance Liquid Chromatography; RBC, Red Blood Cells; [\textsuperscript{3}H]Hb, tritium labeled hemoglobin; PCA, Perchloric Acid; HETLC, HETLC; PBS, phosphate-buffered saline; High Efficiency Thin Layer Chromatography; SDS, Sodium Dodecyl Sulfate; ATZ, 3-amino-1,2,4-triazole.

KEYWORDS: Free Radicals - Proteasome - H\textsubscript{2}O\textsubscript{2} - Oxidative Stress - Dityrosine - Proteolysis - Red Blood Cells - Multicatalytic Proteinase Complex - Oxyhemoglobin
INTRODUCTION

Oxyhemoglobin, the major soluble protein of RBC, undergoes a slow autooxidation producing superoxide anion (ref. 1; eq. 1) which, in the presence of intracellular superoxide dismutase, yields H₂O₂ (eq. 2).

\[
\text{[Fe}^{II}...\text{O}_2] + \text{[Fe}^{III}...\text{O}_2\cdot] \leftrightarrow \text{[Fe}^{III}] + \text{O}_2\cdot^- \quad [1]
\]

\[
2 \text{O}_2\cdot^- + 2 \text{H}^+ \rightarrow \text{H}_2\text{O}_2 + \text{O}_2 \quad [2]
\]

Therefore, hemoglobin is constantly exposed to an intracellular flux of H₂O₂ (generated from its autoxidation or from other intracellular sources; 2) as well as to an extracellular flux, due to the high permeability of this metabolite (3). Exposure of oxyhemoglobin to H₂O₂ leads to oxidative modifications which have been proposed as selective signals for proteolysis in both erythrocytes and reticulocytes by the proteasome (4).

Major interest has been focused on the interaction of H₂O₂ with myoglobin or hemoglobin, and on the possible role of the ferryl species derived from them in the development of several pathophysiological processes. Ferryl species are strong oxidants for several biomolecules including: vitamin E (or its soluble analog Trolox-C (5,6), vitamin C (7,8), cholesterol (9), catecholamines (10), lipoproteins (11), and membrane lipids (12,13). Ferryl hemoglobin has two-oxidizing equivalents above methemoglobin: one oxidizing equivalent is retained in the oxoferryl moiety where the iron has a valence state of +4, and the other oxidizing equivalent is retained in the form of a protein radical, centered on an aromatic residue (14-18), which decays rapidly by pathways not yet elucidated. One of these amino acid radicals in ferrylmyoglobin was identified as a tyrosyl radical, which is dissipated by pathways involving intermolecular diradical cross-linking of Tyr-103 to Tyr-151 of sperm whale myoglobin (19), and covalent binding of the heme group to the protein (20).

We have previously reported that the production of tyrosine oxidation products is correlated with the proteolytic degradation of hemoglobin by proteasome (21). In the present paper, we studied the formation of tyrosine oxidation products after the oxidative modification of oxyhemoglobin solutions and intact RBC. Oxyhemoglobin and RBC were exposed to a continuous flow rate (or flux) of H₂O₂ produced by the glucose-glucose oxidase system, which resembles a physiological condition. The aims of this work were [a] to characterize these oxidation products, [b] to provide a mechanism to explain their formation, and [c] to test their feasibility as specific markers for selective proteolysis.
MATERIALS AND METHODS

Chemicals and Biochemicals

Pronase, catalase, L-DOPA, dopamine, L-tyrosine, sodium dithionite, perchloric acid, glucose, H₂O₂ and methemoglobin were purchased from Sigma Chemical Co. (St. Louis, MO). Horseradish peroxidase and glucose oxidase were obtained from Boehringer Mannheim. All other reagents were of analytical grade.

Preparation of oxyhemoglobin: Fresh oxyhemoglobin was prepared daily from bovine methemoglobin (2x crystallized) by using sodium dithionite in a 2-fold molar excess in deareated 10 mM potassium phosphate buffer (pH 7.8; ref. 22). Tritium-labeled hemoglobin ([³H]Hb) was prepared as previously described (4,21).

Synthesis of dityrosine standards: Dityrosine was prepared by the horseradish peroxidase catalyzed oxidation of L-tyrosine according to Gross and Sizer (23) and Tew and Montellano (19). The reaction mixture contained 25 mM L-tyrosine and 3 µM horseradish peroxidase in 50 mM phosphate buffer (pH 7.6). Hydrogen peroxide (final concentration 2 mM) was added sequentially during 1 h of incubation at 37°C. The resulting mixture was centrifuged through a Centricom™ tube (molecular weight cut-off of 10k) to remove the enzyme. Water from the resulting bottom filtrate was removed on a rotary evaporator and the remaining residue was dissolved in ethanol and chromatographed on 20 x 20 cm preparative (250 µm) silica thin layer plates. The plates were developed with butanol:water:acetic acid 4:1:2 (V/V/V). The plates were exposed to NH₄OH and the blue fluorescent band due to the tyrosine dimer (R_f = 0.26) was excised and extracted with methanol. Crude dityrosine was obtained after removing the solvent by bubbling with N₂. This crude dityrosine was applied to a 20 x 20 cm preparative UNIPLATE (Analtech) silica thin layer plate with organic bounder and eluted with the same system as before. The blue fluorescent band due to dityrosine (R_f 0.26) was excised and extracted with methanol, which was then removed by bubbling with N₂. The white powder was dissolved in acetonitrile/H₂O (70/20) and assessed for purity on an HPLC C-18 column, eluted isocratically with 93% acetonitrile and 7% water; both containing 0.1% trifluoroacetic acid.

Incubation conditions

Fresh human RBC were collected as previously reported (24) from healthy donors. Glucose oxidase was placed in dialysis bags (molecular-weight cut-off of 3,500) which were added just prior to experiments to different flasks containing either 0.2 mM oxyhemoglobin or 5 % (V/V) intact RBC, in 5 mM glucose and Krebs-Ringer phosphate buffer (pH 7.4) at 37°C. The rate of H₂O₂ production
was measured spectrophotometrically at 402-417 nm in a Shimadzu UV-3000 double-beam, dual-wavelength spectrophotometer using the horseradish peroxidase assay according to Boveris et al. (25). The presence of the dialysis bag did not affect significantly the rate of hydrogen peroxide production by the glucose-glucose oxidase system. After 30 min of incubation the dialysis bags were removed and the samples were precipitated with 0.54 M perchloric acid (PCA). The samples were centrifuged at 3,000 rpm for 10 min, and the supernatants were neutralized with 2 M KOH. The crystals of KClO₄ formed were removed by centrifugation. An aliquot of the neutralized PCA-supernatant was diluted with 0.5 M HEPES buffer (pH 9.0) and the fluorescence intensity was measured in an Aminco-Bowman spectrofluorometer, using quinine sulfate as a standard.

**High-performance liquid chromatography of the pronase-treated PCA-supernatants**

Protein fragments present in the neutralized PCA-supernatants were hydrolyzed with 10 µg/ml pronase for 24 h. Pronase was separated by centrifugation in Centricom™ tubes (molecular weight cut-off of 10k). The hydrolysates were chromatographed by high performance liquid chromatography (HPLC) on a Novapak C-18 column, and were eluted with a linear gradient (flow rate 0.8 ml/min) from 0 to 60% of solvent B in 60 min. Solvent A was 0.1% trifluoroacetic acid in water/methanol (75/25, V/V) and solvent B was 0.1% trifluoroacetic acid in acetonitrile/water (80/20; V/V). The eluent was monitored with a Shimadzu fluorescence detector (283 and 315 nm, excitation and emission wavelengths, respectively). The resulting profiles were compared to L-DOPA and dopamine standards purchased from Sigma Chemical Co. or synthesized in the laboratory with tyrosinase (26).
RESULTS AND DISCUSSION

Characterization of tyrosine oxidation products released by fragmentation of oxyhemoglobin after exposure to a flux of H$_2$O$_2$

It is well known that modification of protein conformation and oxidation of amino acids occurs when proteins are exposed to H$_2$O$_2$. Indeed, hemoglobin is irreversibly oxidized during incubation with H$_2$O$_2$ (21,24), yet the oxidation products formed have not been well characterized. One purpose of this study was to identify oxidation products from oxyhemoglobin treated with H$_2$O$_2$. Consequently, bovine oxyhemoglobin (0.2 mM) was exposed to a flux of H$_2$O$_2$ (120 µM/min), generated by the glucose-glucose oxidase system for 30 min at 37°C, and after this treatment the protein samples were precipitated with PCA and neutralized (as described in the Materials & Methods section).

The resulting neutralized supernatants exhibited a fluorescence intensity (excitation and emission wavelengths, 315 nm and 410 nm, respectively), with the following characteristics: [a] fluorescence intensity increased essentially linearly with both the rate of H$_2$O$_2$ generation (Fig. 1A) and the concentration of oxyhemoglobin used (Fig. 1B); [b] the increased fluorescence did not occur in the presence of 10,000 units of catalase (Fig. 1A & B); [c] the fluorescence was still observed after substituting perchloric acid precipitation with a centrifugation step in Centricom™ tubes (molecular weight cut-off of 3k), excluding the possibility of a direct oxidation of protein by the acid treatment (Fig. 1A & B).

These results indicate that the fluorescence observed derived directly from protein fragments (MW < 3 kDa) which contained oxidatively modified amino acids. Indeed, experiments carried out with [³H]-oxyhemoglobin revealed a limited protein fragmentation (1-3%, calculated as percentage of acid-soluble counts), suggesting that a minor portion of the total protein was contributing to the fluorescence intensity (Fig. 2A). A linear relationship ($r = 0.98$) was obtained between fluorescamine-reactive amino groups and the percentage of acid-soluble counts (Fig. 2B).

Further studies were performed to characterize the oxidation products present in the neutralized-PCA supernatants, by UV and fluorescence spectroscopies. The absorption maximum of the neutralized PCA-supernatants in acid solution was found to occur at 283 nm compared with 275 nm for pure tyrosine. In alkaline solution, the maximum shifted to 320 nm and the absorption increased on shifting from pH 2.0 to pH 9.0. The pH at which this bathochromic effect occurred was around 7.2 (data not shown). The pH-dependence of the fluorescent and absorption spectra suggested the presence of a phenolic group with different characteristic from the tyrosine-hydroxyl group ($pK_a = 10.0$).
The pH-dependence of the fluorescence intensity of the PCA-supernatants (Fig. 3A) and tyrosine (Fig. 3B) revealed that only the PCA-supernatants exhibited an increase in fluorescence intensity when excited at 315 nm. If the excitation wavelength was 283 nm, the PCA-supernatants showed an increase in fluorescence intensity above pH 8.0 (Fig. 3A), while tyrosine exhibited decreasing fluorescence intensity at higher pH values (Fig. 3B). The decrease found near pH 9.0 for tyrosine must be due to the dissociation of the amino group (pKₐ = 9.11).

The emission spectrum of the PCA-supernatant (Fig. 3A inset) showed a small increase in fluorescence intensity at both 312 nm and 425 nm emission on going from pH 5 to pH 11, while tyrosine showed a decrease in fluorescence intensity under the same experimental conditions (Fig. 3B inset). These results are in agreement with the production of oxidation products which are different from pure tyrosine, or peptides containing unmodified tyrosine.

High performance liquid chromatography analysis with fluorescent detection of both pronase-treated PCA-supernatants and of pronase-treated hemoglobin pointed to the occurrence of products derived from oxidatively modified tyrosine. Coelution of the products in the pronase-treated PCA-supernatants with synthetic standards, indicated that dopamine, dopamine quinone, and 5,6-dihydroxyindol were the major products of tyrosine oxidation present in the fragments (Table I), whereas DOPA, and 5,6-dihydroxy-3-oxo-indol, were minor products (data not shown). These products were further characterized by using different chromatographic conditions suitable for dopachrome-related products using electrochemical detection (26-27). Importantly, no dityrosine was detectable in pronase digests of the fragments, whereas dityrosine was clearly present in pronase digests of intact Hb (Table I).

**Characterization of tyrosine oxidation products released by proteolysis of oxyhemoglobin after exposure to a flux of H₂O₂**

Dityrosine, along with the other oxidation products already characterized, was identified by HPLC in H₂O₂-treated, and pronase digested oxyhemoglobin (free of fragments) (Table I). No free dityrosine was measured in the neutralized-PCA supernatants without using a proteolytic enzyme (*i.e.*, trypsin, chymotrypsin, pronase, RBC Fraction II) to release it from the H₂O₂-treated oxyhemoglobin (data not shown).

The pronase hydrolysate so obtained and applied to a high efficiency thin layer chromatography (HETLC) plate resulted in one fluorescence spot with an Rₜ value coincident with that of synthetic dityrosine (Fig. 4A). The fluorescence of the compound is rather weak in the presence of acetic acid but it becomes brilliant after exposure of the plate to ammonia vapour, and the presence of a phenolic
group was indicated by the positive reaction of the spot with Folin's phosphomolybdic acid reagent (Fig. 4A).

SDS-PAGE analysis of oxyhemoglobin after incubation with H₂O₂ (Fig. 4B) showed that the monomeric protein (Mr = 14,000) is partially converted to dimeric (Mr = 25-30,000), and trimeric (Mr = 42,000) products. Laser scanning of the bands indicates that approximately 10% (Fig. 4B; lane d) to 20% (Fig. 4B; lane e) of the protein is converted to dimer upon exposure of the protein to a bolus addition of H₂O₂. Although, under the present experimental conditions we are not able to distinguish the composition of the dimer (i.e. type α₂, ß₂ or αß), the formation of a tyrosine radical was confirmed by EPR spectroscopy (see below).

**Chemical requirements and characterization of the oxidant species responsible for the formation of tyrosine oxidation products**

Experiments were carried out to determine the chemical requirements for the production of fluorescent compounds in hydrogen peroxide-treated oxyhemoglobin. The production of fluorescent compounds was dependent on the presence of both H₂O₂ and the heme (Table II) because absence of either H₂O₂ or the heme (in globin or by binding methemoglobin with cyanide) resulted in a negligible fluorescence intensity indicating that the production of the original fluorescent products was halted or that other products with lower quantum yield had been formed. The latter possibility seemed unlikely under our experimental conditions because the UV-vis spectrum of the supernatants from methemoglobin, hydrogen peroxide, and cyanide or globin plus hydrogen peroxide did not show the presence of products different from those obtained with methemoglobin plus hydrogen peroxide, and no new peaks were found by analyzing these supernatants by HPLC. Thus the majority of Hb oxidation products appear to be formed during the first H₂O₂/oxyhemoglobin interaction, and the remaining products must be formed during the subsequent reactions of methemoglobin with H₂O₂.

The small amount of fluorescent compounds found in the globin/H₂O₂ system (Table II) indicates that the majority of products formed in the Hb/H₂O₂ system are not the result of a direct oxidation of amino acids by H₂O₂, and that another oxidizing species (either the oxoferryl moiety or an oxidant species derived from it) is responsible for the formation of tyrosine oxidation products. In our model system, the possible candidates are: a) hydroxyl radical (formed during the homolytic cleavage of H₂O₂ by oxyhemoglobin or deoxyhemoglobin); b) oxoferryl moiety of ferrylhemoglobin [which with a redox potential of about 1.4 V (28) should be able to effect a hydrogen abstraction, although the latter may be less efficient than hydroxyl radical because of a restricted steric hindrance]; and c) a protein-centered radical.
A role for ferrylhemoglobin, as the oxidant species during the oxidation of oxyhemoglobin by a continuous flux of H\textsubscript{2}O\textsubscript{2}, is substantiated by the following experimental results: 1) a transient visible spectrum ascribed to ferrylhemoglobin (Fig. 5A); 2) an EPR spectrum of the protein radical (Fig. 5B); and 3) the lack of a DMPO-OH adduct EPR signal. Each of these results is considered in detail below. The oxidation of 0.2 mM oxyhemoglobin by the glucose-glucose oxidase system (120 µM H\textsubscript{2}O\textsubscript{2}/min) produced spectral changes in the visible region consisting of the transient appearance of two absorption peaks at 545 and 580 nm, along with a decrease in the absorbance at 540 and 575.6 nm (Fig. 5A). At 60 min the resulting spectral profile showed a maximum absorbance at 630 nm consistent with that of native methemoglobin. These spectral changes may be understood in terms of a transition from oxyhemoglobin to methemoglobin, which proceeds through ferrylhemoglobin as an intermediate, as was discussed previously (24). The contribution of a protein radical cannot be evaluated spectrophotometrically because HX-Fe\textsuperscript{IV}-OH and \cdotX-Fe\textsuperscript{IV}-OH are indistinguishable by visible spectroscopy. A protein radical EPR signal (Fig. 5B), similar to the tyrosine radical signal obtained with horse myoglobin and H\textsubscript{2}O\textsubscript{2} (4-5) and with ribonucleoside diphosphate reductase (29), was detected by mixing oxyhemoglobin and H\textsubscript{2}O\textsubscript{2} (in a continuous flow). In contrast, no signal ascribable to the hydroxyl radical was obtained with 0.2 mM oxyhemoglobin, glucose-glucose oxidase (120 µM H\textsubscript{2}O\textsubscript{2}/min), and the spin trap DMPO at concentrations of 80-200 mM (data not shown). Although hydroxyl radical formation from hemoglobin-catalyzed homolytic cleavage of H\textsubscript{2}O\textsubscript{2} has been previously reported, subsequent work suggests that the radical detected was formed by secondary reactions of the peroxide with iron released from the oxidatively damaged heme group (30-31).

The Hb protein radical may well be localized in the α-Tyr-42 in bovine hemoglobin, which is close to the heme group and exposed to the surrounding medium (Fig. 6).

*Mechanism of formation of tyrosine oxidation products and dityrosine in H\textsubscript{2}O\textsubscript{2}-treated oxyhemoglobin*

It has been reported that the protein radical formed in ferrylmyoglobin could decay a) by tyrosine addition to the heme group, as described by Catalano et al. (20) for myoglobin, b) by dityrosine cross-link formation as in sperm whale myoglobin (19), or c) by an oxygen addition to the aromatic radical in either hemoglobin or myoglobin (32-33). The latter mechanism, oxygen addition to the aromatic radical, would yield a peroxyl tyrosine radical, which could be proposed as the initial step in the formation of oxidation products. This protein peroxyl radical was proposed to rationalize the epoxidation of styrene by hemoglobin and myoglobin (34), however, recent reports by the same group...
(35) and by others (36-37) seem to rule out this possibility, and an iron-dioxygen complex [HX-Fe^{II}-O-O^-] was proposed as an alternative oxidizing species. The starting step for the generation of these products (or other amino acid-derived oxidation products) could be accomplished by any of the oxidizing species discussed above (namely the protein radical, the oxoferryl moiety, or the iron-dioxygen complex). The formation of Tyr oxidation products might involve cyclization, decarboxylation, and further oxidations steps on either the apoprotein or fragments released from the protein. The chemistry of the reaction hemoglobin/H_2O_2 is complicated because not only several oxidizing species might contribute to the formation of oxidized products, but also the dynamic changes involved in this process (i.e., the translocation of the protein radical throughout the protein structure (38) and the transfer of the radical character from carbon- to oxygen-centered radicals by oxygen addition to amino acid radicals) may obscure the possibility of defining a clear, accurate mechanism for the formation of these oxidized products.

The formation of the tyrosine oxidation products discussed above, can be explained by the participation of either one or two monomers of hemoglobin (α or β subunit), while the formation of dityrosine probably involves two monomeric molecules of hemoglobin, joined by intermolecular cross-linking, because intramolecular cross-linking among tyrosine residues located in the same molecule is rather difficult (Fig. 6). The mechanism proposed for the production of dityrosine (Fig. 7) includes the formation of tyrosyl radicals, subsequent diradical reaction and finally enolization.

**Tyrosine oxidation products are formed and released in RBC exposed to a H_2O_2 flux**

Bovine, rabbit, or human intact RBC (5%, V/V) exposed to different flow rates of H_2O_2 for 30 min, showed an increase in fluorescence intensity at 320/425 nm in the neutralized-PCA supernatants (Fig. 8). Dityrosine contribution to this increased fluorescence was minimum (1-2%; calculated using pure dityrosine as standard), whereas the main contribution was from other oxidation products of tyrosine identified as described above. This minimum production of dityrosine could be attributed to the generation of dityrosine from free tyrosine in the RBC (39; see below). A major contribution of dityrosine to the total increased fluorescence (about 20%) was obtained after long periods of incubation (more than 4 h) of intact RBC, or by the addition of exogenous proteolytic enzymes (i.e., pronase) to lysed cells (data not shown).

Bearing in mind that ferrylhemoglobin is a highly oxidizing species similar to Compound II of peroxidases, the formation of free dityrosine in RBC exposed to H_2O_2 can be explained by [a] a peroxidatic activity of ferrylhemoglobin using tyrosine (from the intracellular pool of amino acids) as a substrate or [b] proteolytic release of dityrosine previously formed in the oxidatively modified
When a reaction mixture of 0.2 mM oxyhemoglobin and 1.7 mM H$_2$O$_2$ was supplemented with 25 mM L-tyrosine, in order to compete with any molecule of hemoglobin, the formation of free dityrosine was measured (Fig. 9A). Dityrosine was identified by HPLC, using synthetic dityrosine standards. For comparison horseradish peroxidase was used in parallel experiments, and was found to be to be six-times (per heme) more efficient than hemoglobin in catalyzing the same reaction (Fig. 9A inset).

Lysed RBC incubated with H$_2$O$_2$ and 25 mM L-tyrosine also produced free dityrosine up to a protein concentration of 1.5 mg/ml. At higher concentrations of protein, the amount of dityrosine produced decreased to almost zero (Fig. 9B). Since catalase concentration increased with increasing concentration of protein additions, the experiment was repeated in the presence of 0.1 M 3-aminotriazole (aminotriazole or ATZ), an inhibitor of the catalase-hydrogen peroxide Complex I (40-42). The fluorescence intensity measured in lysed RBC with aminotriazole was comparable to the intensity measured with hemoglobin alone (Fig. 9A) at 25 mM tyrosine. Aminotriazole did not exert any effect on dityrosine production by Hb (not shown). This observation was supported by specific inhibition of catalase-hydrogen peroxide Complex I through the binding of ATZ to a His residue in the peptide VVHAK (43); the target sequence is highly conserved among catalases from different species, not present in hemoglobin or myoglobin.

The results shown in Fig. 9B indicate that RBC experiencing a flux of H$_2$O$_2$ do not produce dityrosine from free intracellular tyrosine, because the minimum tyrosine concentration needed to obtain measurable amounts of dityrosine in lysed RBC (without aminotriazole) is $5 \times 10^{-4}$ M (Fig. 9B, inset); a concentration 8-times higher than the actual intracellular concentration of free tyrosine corrected by the volume of water excluded by hemoglobin (60-70 µM)$^1$.

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$^1$ Given the volume of a spherical molecule ($4/3 \pi r^3$), the radius of Hb ($r = 2.75 \times 10^{-11}$ cm; 44), the volume per molecule can be calculated as $8.7 \times 10^{-20}$ cm$^3$. Considering that [Hb] in RBC is 34 g/100 ml, MW = 64,500, and the average volume of RBC for an adult man is $87 \mu$m$^3$ (44-45) then the volume for unsolvated Hb molecules contained in a RBC is $2.4 \times 10^{-11}$ cm$^3$. This indicates that unsolvated Hb occupies 27.6% of the total volume of RBC. The partial specific volume for Hb can be estimated to be close to that of myoglobin ($0.741$ g/cm$^3$; 46) given the homologies between the similarities between these two proteins. Thus, the partial specific volume of Hb ($\upsilon_2$) plus the specific volume of water ($\upsilon_1 = 1.00$ cm$^3$/g) times the amount of hydration ($\delta_1$) can be calculated from the following equation: $(ff/f_o)^3 \times \upsilon_2 = \upsilon_2 + \delta_1 \upsilon_1$, where $ff/f_o = 1.105$ (46). The volume of solvated Hb is 0.999 cm$^3$/g, representing a 34% of the total RBC volume (0.999 cm$^3$/g).
Further evidence that dityrosine was originated from proteolytic release of this dimer previously formed in the oxidatively modified protein was previously observed by using proteolytic inhibitors to prevent dityrosine release (21).

CONCLUSIONS

The aims of the present study were as follows: a) to characterize the oxidation products formed after exposing oxyhemoglobin to a flux of H2O2; b) to provide a plausible mechanism for the formation of these products; and c) to test the feasibility of dityrosine measurements as markers for oxidative stress and for selective proteolysis.

Several oxidation products of tyrosine were obtained after exposing oxyhemoglobin or intact RBC to a flux of H2O2. These products were released by fragmentation or by proteolysis of the hydrogen peroxide-treated oxyhemoglobin (this paper and ref. 21) after complicated reactions which involved cyclization, H-abstraction, oxidation and interaction with proximal amino acids.

Previously it was shown that the multicatalytic proteinase complex, proteasome, recognizes and degrades oxidatively modified hemoglobin in RBC by a mechanism that appears to rely on increased hydrophobicity and that dityrosine is released in the process (4,21,47). Among the many oxidation products of tyrosine, dityrosine should be considered a specific marker for selective proteolysis (although a minor product in quantitative terms) because its release occurs only after an oxidative stress (exposure to H2O2) and after proteolysis. Dityrosine release can be measured by HPLC with (48) or without (this paper and ref. 49) derivatization, or by HETLC, taking into account that the latter technique is at least 100-times less sensitive than the other two. Moreover, dityrosine has the advantage over the other oxidation products (which are sensitive to oxygen and to high pH) of being a stable compound because once the 3′-3′ carbon-carbon bond is formed it is resistant to hydrolysis by all other lytic enzymes in the red cell (21). Dityrosine can also be measured quantitatively and definitively by stable isotope dilution gas chromatography-mass spectrometry has been ably demonstrated by Heinecke and co workers (50-51). Unfortunately, the expense and expertise required for stable isotope dilution gas chromatography-mass spectrometry analysis (50-51) may put this technique beyond the reach of many investigators.

\[ \text{cm}^3/\text{g} \times 2.96 \times 10^{-11} \text{ g Hb} \]. If we correct the concentration of free tyrosine in adult RBC (mean: 42 µM; 39) considering the free water, then the concentration would be equal to 64 µM, about 8-times lower than the threshold for dityrosine production.
Although the mechanism for dityrosine formation in myoglobin and hemoglobin seems to be similar on a molecular basis, dityrosine cross-linking in myoglobin only occurs in sperm whale myoglobin, which has an essential Tyr-151 (19), and as a consequence dityrosine release from cardiac human myoglobin, after after ischemia-reperfusion injury, cannot be considered as a possible marker for oxidative stress. On the other hand, the release of dityrosine from hemoglobin (bovine, human or rabbit) seems to be independent of the hemoprotein source under our experimental conditions, and several reports have shown the formation of dityrosine in different proteins exposed to a variety of oxidants (UV light, γ-irradiation, oxygen radicals; 52 and references therein), supporting a wider use of dityrosine release as a marker for oxidative stress and/or selective proteolysis. Although the dityrosine production and release demonstrated in this paper, and previously (21,24) may not be quantitatively as widespread as production of carbonyl groups in oxidized proteins (53-54) we suggest that the great specificity of dityrosine may be of particular value.
REFERENCES

TABLE I

Tyrosine oxidation products in hydrogen peroxide-treated hemoglobin

<table>
<thead>
<tr>
<th>Tyrosine oxidation products</th>
<th>Pronase Digested Dopamine quinone</th>
<th>Dopamine</th>
<th>Dihydroxyindol</th>
<th>Dityrosine</th>
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<tr>
<td></td>
<td>(Peak height ratio Product/Tyrosine)</td>
<td>(1.34 min)</td>
<td>(3.27 min)</td>
<td>(7.38 min)</td>
</tr>
<tr>
<td>Hemoglobin fragments</td>
<td>1.07</td>
<td>0.60</td>
<td>1.38</td>
<td>-</td>
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<tr>
<td>Hemoglobin</td>
<td>3.22</td>
<td>4.84</td>
<td>1.46</td>
<td>0.92</td>
</tr>
</tbody>
</table>

Oxyhemoglobin (0.2 mM) was treated with 7.5 µg/ml glucose oxidase. After 30 min at 37°C, the samples were centrifuged in microconcentrator Centricom™ tubes (3 k molecular weight cut-off). The solution of compounds with molecular weights higher (Hb), or lower (Hb fragments) than 3 k, obtained from the Centricom™ tubes, were treated for 24 h with pronase (10 µg/ml) separately. Measurement of dityrosine: The pronase-digested hemoglobin was chromatographed on a Bondapak C-18 column (5µ, 30 cm) and eluted with the following elution program: Solvent A was run with a flow rate of 0.8 ml/min for 10 min; from 10 min until 20 min a gradient of solvent B from 0-4% was run; solvent B (100%) was run for further 20 min. Solvent A; Methanol/H2O (25/75, V/V); Solvent B; Acetonitrile/H2O (80/20, V/V), both solvents with 0.1% (V/V) trifluoroacetic acid. The detection was carried out with a Shimadzu fluorodetector at 284 nm excitation and 325 nm emission. Tyrosine and dityrosine were identified by the following experimental procedures: (a) comparison of the retention times of pure tyrosine and dityrosine, and (b) by coinjection of pure standards with the samples.

Measurement of tyrosine oxidation products: The pronase-digested samples of hemoglobin fragments were applied to an HPLC-column Bondapak C-18 (5µ, 30 cm) using as mobile phase 30 mM HPO4, 0.1 mM EDTA, and 20% methanol (pH 2.79) at a flow rate of 1.5 ml/min. Fluorescence detection was at 308 nm excitation and 395 nm emission. Dopamine, dopaminequinone and 5,6-dihydroxyindol were identified by comparing retention times with standards synthesized from L-dopamine as follows: dopamine (1 mM) was treated with mushroom tyrosinase or polyphenol oxidase (10 µg/ml) in 0.5 M sodium phosphate buffer, pH 6.5 at 0-4°C. After 15 min of incubation, aliquots of the reaction mixture were diluted 1:10 with mobile phase.
TABLE II

Fluorescent products formed from methemoglobin or globin
treated with H₂O₂

<table>
<thead>
<tr>
<th>Additions</th>
<th>Fluorescence intensity ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methemoglobin + H₂O₂</td>
<td>2.18</td>
</tr>
<tr>
<td>Methemoglobin + KCN</td>
<td>1.00</td>
</tr>
<tr>
<td>Methemoglobin + H₂O₂ + KCN</td>
<td>1.30</td>
</tr>
<tr>
<td>Globin + H₂O₂</td>
<td>1.10</td>
</tr>
</tbody>
</table>

Assay conditions: 0.2 mM globin or hemoglobin in phosphate buffered saline solution (pH 7.4) was treated with 2 mM H₂O₂ for 30 min at room temperature. The fluorescence intensity of the neutralized supernatants was measured as described in Materials & Methods. A ratio of the fluorescence intensities (excitation and emission wavelengths were 300 and 410 nm, respectively) was obtained by dividing the intensity of protein plus the corresponding addition, by the fluorescence intensity of methemoglobin alone.
FIGURE LEGENDS

Figure 1  
*Fluorescent products are formed upon H₂O₂-treatment of oxyhemoglobin.*

Panel A: Oxyhemoglobin (0.2 mM) was treated for 30 min with different concentrations of glucose oxidase 0-7.5 µg/ml [in the presence (square) or absence (open and closed circles) of 10,000 I.U. of catalase] in 5 mM glucose plus Krebs-Ringer phosphate buffer (pH 7.4) at 37°C. Following H₂O₂ exposure the remaining intact hemoglobin was removed either by precipitation with PCA (open circles), or by use of Centricom™ tubes (closed circles) with a molecular weight cut-off of 3 kDa (See Materials and Methods). The fluorescence of the neutralized PCA-supernatants and the Centricom™ eluents was measured at the excitation and emission wavelengths 315 and 425 nm respectively, in an Aminco-Bowman spectrofluorometer using quinine sulfate as a standard.

Panel B: Different amounts of oxyhemoglobin were treated with 7.5 µg/ml of glucose oxidase in the buffer described above. The fluorescence measurements were carried out as described in Panel A. In both panels fluorescence intensity is reported in arbitrary fluorescence units (A. U.).

Figure 2  
*Hemoglobin undergoes minor fragmentation after exposure to a flux of H₂O₂*

Panel A: [³H]-Hemoglobin (0.33 mg/ml) was treated with different concentrations of glucose oxidase in a solution containing 5 mM glucose and Krebs-Ringer phosphate buffer (pH 7.4). After 30 min, hemoglobin fragmentation was measured either by the formation of acid-soluble counts (closed circles) or by increase in fluorescamine reactive (acid-soluble) free amino groups (open circles).

Panel B: Correlation of fluorescamine reactive amino groups and acid-soluble counts ($r = 0.98$). Fluorescence is reported in arbitrary units (A. U.).

Figure 3  
*The pH dependence of fluorescence profiles from PCA supernatants of H₂O₂-treated hemoglobin, and pure tyrosine*

The neutralized PCA supernatant was obtained from bovine oxyhemoglobin (0.2 mM) after treatment with 2.0 µg/ml glucose oxidase (32 µM H₂O₂/min) as described in Materials & Methods.

Panel A: The pH profile was carried out with 0.2 M of either sodium phosphate (pH 5-8) or HEPES buffer (pH 8-11). The fluorescence was measured in an Aminco-Bowman spectrofluorometer with quinine sulfate as a standard, at the following pairs of wavelengths: $\lambda_{\text{excitation}}$ 283 nm and $\lambda_{\text{emission}}$ 312 nm (closed circles) and, $\lambda_{\text{excitation}}$ 315 nm and $\lambda_{\text{emission}}$ 425 nm (open circles).

Panel B: As described for Panel A but using 0.1 mM pure tyrosine. Inset: Emission
spectra of PCA-neutralized supernatant (A) or pure tyrosine (B) using λ<sub>excitation</sub> 283 nm at pH 5 (solid line) or pH 7.4 (dotted line).

Figure 4 **High-efficiency thin layer chromatography (HETLC) of H<sub>2</sub>O<sub>2</sub>-treated, pronase-digested oxyhemoglobin and SDS-PAGE demonstrating covalent cross-linking of hemoglobin by H<sub>2</sub>O<sub>2**

**Panel A:** Oxyhemoglobin (0.2 mM) was treated with 120 µM/min H<sub>2</sub>O<sub>2</sub> in a solution containing 5 mM glucose and Krebs-Ringer phosphate buffer (pH 7.4). After 30 min of exposure, control and treated samples were freed of fragments by centrifuging in Centricom™ tubes (molecular-weight cut-off of 30k). Intact protein at the top of the filter was hydrolyzed for 24 h with 10 µg/ml pronase. The hydrolysates were lyophilized in a Speed-Vac centrifuge and the resulting powder was dissolved in methanol. Aliquots of each sample were applied to HETLC plates consisting of normal phase silica gel with organic binder (Analtech, Inc. # 58077) and separated using n-butanol:water:acetic acid (4:1:2) as the solvent. The plates were developed either by illumination with UV light (previous exposure to NH<sub>3</sub> vapour; not shown) or sprayed with phosphomolibdic acid reagent for phenols. The samples were compared to recrystallized tyrosine and pure dityrosine (R<sub>f</sub> tyrosine = 0.54; R<sub>f</sub> dityrosine = 0.33). Lane A shows unmodified hemoglobin whereas lane B shows hydrogen peroxide-treated hemoglobin. A pure tyrosine standard was run in lane C and a purified dityrosine standard is shown in lane D.

**Panel B:** Bovine methemoglobin (10 µM) in phosphate buffered saline solution (pH 7.4) was exposed to H<sub>2</sub>O<sub>2</sub> at the following molar ratios: 0, 1, 5, 10 and 20. The molar ratios used for these bolus H<sub>2</sub>O<sub>2</sub> treatments exactly match the total H<sub>2</sub>O<sub>2</sub> exposure experienced during the 30 min incubations with glucose oxidase reported in Fig. 1. After 30 min of incubation at 37°C, catalase (7,600 IU/ml) was added to remove any possible excess of peroxide. Aliquots from each tube were boiled for 10 min in the presence of dithiotreitol and sodium dodecyl sulphate (SDS). The samples were next separated in a 12.5% homogeneous SDS-electrophoresis gel (Pharmacia, “Phast System”) and visualized with silver stain. Lane a, low molecular weight markers; lane b, untreated hemoglobin; lanes c through f, hemoglobin treated with 10, 50,100 and 200 µM H<sub>2</sub>O<sub>2</sub>. The approximately 1% of protein fragmentation expected from the results of Fig. 1 is not evident in the gel shown since the samples applied would have only 0.6 ng of protein fragments, which is below the silver-stain detection limit. The molecular markers were used to calibrate a standard curve of R<sub>f</sub> vs. log M<sub>r</sub> for which the experimentally obtained linear correlation coefficient was 0.99 (p < 0.01).
**Figure 5** Absorption spectrum of oxyhemoglobin exposed to \( H_2O_2 \) from the glucose-glucose oxidase system and EPR spectrum of the protein radical formed during the oxidation of oxyhemoglobin by \( H_2O_2 \).

**Panel A:** Oxyhemoglobin (0.2 mM) was treated with 10 mM glucose and 7.5 µg glucose oxidase/ml in Krebs-Ringer phosphate buffer (pH 7.4) at 37°C. A decrease at 540 and 577 nm (disappearance of oxyhemoglobin) and a transient increase at 545 and 580 nm (formation of ferrylhemoglobin) were observed. The increase at 630 nm was consistent with the occurrence of methemoglobin (dotted line) at 60 min. Repetitive scans were recorded every 5 min for 40 min.

**Panel B:** The EPR spectrum was recorded on a Bruker ECS 106 spectrometer equipped with a flow apparatus. The reaction was carried out in PBS buffer (pH 7.4) at 25°C. The oxyhemoglobin and \( H_2O_2 \) solutions, both in the same PBS buffer, were mixed at 10 ml x min\(^{-1} \) in equal volumes with the flow apparatus, and the final concentrations of oxyhemoglobin and \( H_2O_2 \) were 0.2 mM and 2 mM, respectively, in the EPR reaction chamber. Instrument settings: receiver gain, \( 1 \times 10^6 \); microwave power, 20 mW; microwave frequency, 9.81 GHz; modulation amplitude, 1.995 G; time constant, 327.68 ms; scan time, 2.8 min.

**Figure 6** Computer representation of hemoglobin

The amino acids located in the surface of the protein were represented in wireframe (grey); the heme was depicted in sticks (grey), heme iron in yellow, and 42-Tyr in blue.

**Figure 7** Proposed mechanism for dityrosine formation from methemoglobin and \( H_2O_2 \).

**Figure 8** Red blood cells exposed to \( H_2O_2 \) from the glucose-glucose oxidase system release tyrosine oxidation products.

Bovine, rabbit and human RBC (5 %, V/V) were treated with different concentrations of glucose oxidase in 10 mM glucose plus Krebs-Ringer phosphate buffer (pH 7.4) at 37°C. After 30 min of incubation, the fluorescence intensity (320 nm excitation/425 emission) was measured in the neutralized PCA-supernatants as described previously.

**Figure 9** Dityrosine formation by horseradish peroxidase, hemoglobin and lysed blood cells.
**Panel A:** L-tyrosine (25 mM) in 50 mM sodium phosphate buffer (pH 7.8) was treated with different concentrations of either horse radish peroxidase or oxyhemoglobin in the presence of 2 mM H2O2. After 1 h of incubation at 37°C, the samples were centrifuged in Centricom™ tubes (molecular-weight cut off of 10k) to remove the proteins. The fluorescence intensity of the samples was measured at 320 nm and 425 nm, excitation and emission wavelengths, respectively, after adjusting the pH to 9.0. The correspondent fluorescence units were read against a calibration curve made with pure dityrosine (extinction coefficient = 4.2 M⁻¹ cm⁻¹). Other experimental conditions were as described in the legend to Fig. 1. **Inset:** Linear relationship of the dityrosine fluorescence with the concentration of protein ($r_{HRP} = 0.997$, slope 14.19; $r_{Hb} = 0.989$, slope 5.95).

**Panel B:** Human RBC (5 % V/V) were lysed in water (1:10, V/V) and aliquots of the lysate were incubated with 25 mM L-tyrosine and 2 mM H2O2 for 1 h at 37°C in the presence (closed circles) and absence (open circles) of 0.1 M 3-amino-1,2,4-triazole. The fluorescence of the neutralized PCA-supernatants was measured as described in Fig. 1. Other experimental conditions were as described in Materials and Methods. **Inset:** Lysed RBC (0.02 - 10.0 mg protein/ml) were incubated with different concentrations of L-tyrosine and 2 mM H2O2 in 50 mM sodium phosphate buffer (pH 7.4) in the presence (closed circles) and absence (open circles) of 0.1 M aminotriazole.
Fig. 1

Rate of hydrogen peroxide production (μM/min)

Fluorescence intensity (A.U.)

[Glucose oxidase] (μg/ml)

[Oxyhemoglobin] (μM)
Figure 2; Giulivi & Davies
A Neutralized-PCA supernatants

B Tyrosine

Fig. 3; Giulivi & Davies
Giulivi & Davies; Figure 4
A

B

g = 2.0048

Giulivi & Davies; Figure 5
Giulivi and Davies; Figure 7
Fluorescence intensity (A. U.) vs. [Glucose oxidase] (µg/ml)

- Bovine RBC
- Rabbit RBC
- Human RBC

Giulivi and Davies; Figure 8
Fig. 9
Mechanism of hydrogen peroxide-induced formation of dityrosine and tyrosine oxidation products in hemoglobin and red blood cells and their release by proteolysis

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