Mechanism of the Formation and Proteolytic Release of H₂O₂-induced Dityrosine and Tyrosine Oxidation Products in Hemoglobin and Red Blood Cells*

Received for publication, November 27, 2000, and in revised form, February 15, 2001
Published, JBC Papers in Press, April 9, 2001, DOI 10.1074/jbc.M010697200

Cecilia Giulivi‡ and Kelvin J. A. Davies¶¶
From the ‡Department of Chemistry, University of Minnesota, Duluth, Minnesota 55812 and the ¶Ethel Percy Andrus Gerontology Center and Division of Molecular Biology, University of Southern California, Los Angeles, California 90089-0191

Oxyhemoglobin exposed to a continuous flux of H₂O₂ underwent oxidative modifications, including limited 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 that 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 with fluorescence detection or by high efficiency thin layer chromatography. Comparable results, in terms of dityrosine release, were obtained using red blood cells of different sources after exposing them to a flux of H₂O₂. 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.

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

\[
\text{[Fe}^\text{II} \cdots \text{O}_2^\text{−}] + \text{[Fe}^\text{II} \cdots \text{O}_2^\text{−}] \rightarrow \text{[Fe}^\text{II}] + \text{O}_2^\text{−} \quad (\text{Eq. 1})
\]

\[
2\text{O}_2^\text{−} + 2\text{H}^\text{+} \rightarrow \text{H}_2\text{O}_2 + \text{O}_2 \quad (\text{Eq. 2})
\]

Therefore, hemoglobin is constantly exposed to an intracellular flux of H₂O₂ (generated from its autooxidation or from other intracellular sources; Ref. 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 that 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 (i) to characterize these oxidation products, (ii) to provide a mechanism to explain their formation, and (iii) to test their feasibility as specific markers for selective proteolysis.

MATERIALS AND METHODS

Chemicals and Biochemicals

Pronase, catalase, L-3,4-dihydroxyphenylalanine, dopamine, L-tyrosine, sodium dithionite, perchloric acid, glucose, H₂O₂, and methemoglobin were purchased from Sigma. Horseradish peroxidase and glucose oxidase were obtained from Roche Molecular Biochemicals. All other reagents were of analytical grade.

Preparation of Oxyhemoglobin—Fresh oxyhemoglobin was prepared daily from bovine methemoglobin (22 crystallized) by using sodium dithionite in a 2-fold molar excess in deoxygenated 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 μg 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 cutoff of 10,000) 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 × 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 (Rᵢ = 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 × 20-cm preparative UNIPLATE (Analtichem) silica thin layer plate with organic bounder and eluted with the same system as before. The blue fluorescent band due to dityrosine (Rᵢ = 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 a high performance liquid chromatography (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 cutoff 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 actually at 402–417 nm in a Shimadzu UV-3000 double beam, dual wavelength spectrophotometer using the horseradish peroxidase assay.

The resulting neutralized supernatants exhibited a fluorescence intensity (excitation and emission wavelengths, 315 and 410 nm, respectively) with the following characteristics: (i) fluorescence intensity increased essentially linearly with both the rate of H₂O₂ generation (Fig. 1A) and the concentration of oxyhemoglobin used (Fig. 1B); (ii) the increased fluorescence did not occur in the presence of 10,000 units of catalase (Fig. 1, A and B); (iii) the fluorescence was still observed after substituting perchloric acid precipitation with a centrifugation step in Centricom™ tubes (molecular weight cutoff of 3,000), excluding the possibility of a direct oxidation of protein by the acid treatment (Fig. 1, A and B).

These results indicate that the fluorescence observed derived directly from protein fragments (molecular mass < 3 kDa) that 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 contributed 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

**RESULTS AND DISCUSSION**

**Characterization of Tyrosine Oxidation Products Released by Fragmentation of Oxyhemoglobin after Exposure to a Flux of H₂O₂—**It is well known that modification of protein conformation and oxidation of amino acids occurs when proteins are exposed to H₂O₂. Indeed, hemoglobin is irreversibly oxidized during incubation with H₂O₂ (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 H₂O₂. Consequently, bovine oxyhemoglobin (0.2 mM) was exposed to a flux of 120 μM H₂O₂/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 under “Materials and Methods”).

The resulting neutralized supernatants exhibited a fluorescence intensity (excitation and emission wavelengths, 315 and 410 nm, respectively) with the following characteristics: (i) fluorescence intensity increased essentially linearly with both the rate of H₂O₂ generation (Fig. 1A) and the concentration of oxyhemoglobin used (Fig. 1B); (ii) the increased fluorescence did not occur in the presence of 10,000 units of catalase (Fig. 1, A and B); (iii) the fluorescence was still observed after substituting perchloric acid precipitation with a centrifugation step in Centricom™ tubes (molecular weight cutoff of 3,000), excluding the possibility of a direct oxidation of protein by the acid treatment (Fig. 1, A and B).

These results indicate that the fluorescence observed derived directly from protein fragments (molecular mass < 3 kDa) that 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 contributed 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
Fig. 2. Hemoglobin undergoes minor fragmentation after exposure to a flux of \( \text{H}_2\text{O}_2 \). A. \( ^{3}\text{H}\)hemoglobin (0.33 ng/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 (filled circles) or by the increase in fluorescamine-reactive (acid-soluble) free amino groups (open circles). B, correlation of fluorescamine-reactive amino groups and acid-soluble counts (\( r = 0.98 \)). Fluorescence is reported in arbitrary units (A.U.).

The small amount of fluorescent compounds found in the globin-\( \text{H}_2\text{O}_2 \) system (Table II) indicates that the majority of products formed in the \( \text{Hb-}\text{H}_2\text{O}_2 \) system are not the result of a direct oxidation of amino acids by \( \text{H}_2\text{O}_2 \). The remaining products must be formed during the subsequent reactions of methemoglobin with \( \text{H}_2\text{O}_2 \).

Characterization of Tyrosine Oxidation Products Released by Proteolysis of Oxyhemoglobin after Exposure to a Flux of \( \text{H}_2\text{O}_2 \)—Dityrosine, along with the other oxidation products already characterized, was identified by HPLC in \( \text{H}_2\text{O}_2 \)-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 (Table I). No free dityrosine was measured in the neutralized PCA supernatants without using a proteolytic enzyme (Table I). The pH dependence of the fluorescent and absorption spectra suggested the presence of a phenolic group with different characteristics from the tyrosine hydroxyl group (Table I).

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), whereas 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 (pK\(_a\) = 10.0).

The emission spectrum of the PCA supernatant (Fig. 3A, inset) showed a small increase in fluorescence intensity at both 312 and 425 nm emission on going from pH 5 to 11, whereas 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 that 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 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 tyrosine oxidation was not able to distinguish the composition of the dimer (Table I).

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 \( \text{H}_2\text{O}_2 \) and the heme (Table II), because absence of either \( \text{H}_2\text{O}_2 \) 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-visible 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 \( \text{H}_2\text{O}_2 \)-oxyhemoglobin interaction, and the remaining products must be formed during the subsequent reactions of methemoglobin with \( \text{H}_2\text{O}_2 \).

Characterization of Tyrosine Oxidation Products Released by Proteolysis of Oxyhemoglobin after Exposure to a Flux of \( \text{H}_2\text{O}_2 \)—Dityrosine, along with the other oxidation products already characterized, was identified by HPLC in \( \text{H}_2\text{O}_2 \)-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 \( \text{H}_2\text{O}_2 \)-treated oxyhemoglobin (data not shown).

The Pronase hydrolysate so obtained and applied to a high efficiency thin layer chromatography plate resulted in one fluorescence spot with an \( R_t \) 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 vapor, 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-polyacrylamide gel electrophoresis analysis of oxyhemoglobin after incubation with \( \text{H}_2\text{O}_2 \) (Fig. 4B) showed that the monomeric protein (\( M_r = 14,000 \)) is partially converted to dimeric (\( M_r = 25,000-30,000 \)) and trimeric (\( M_r = 42,000 \)) products. Laser scanning of the bands indicates that ~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 \( \text{H}_2\text{O}_2 \). Although under the present experimental conditions we were not able to distinguish the composition of the dimer (i.e. type \( \alpha_2 \beta_2 \), \( \alpha_2 \beta_2 \), or \( \alpha_2 \beta_2 \)), the formation of a tyrosine radical was confirmed by EPR spectroscopy (see below).
Dityrosine in Hemoglobin and Erythrocytes

The absorbance at 540 and 575.6 nm (Fig. 5A) absorption peaks at 545 and 580 nm along with a decrease in the visible region consisting of the transient appearance of two evaluated spectrophotometrically because HX-FeIV-OH and previously (24). The contribution of a protein radical cannot be through ferrylhemoglobin as an intermediate, as was discussed from oxyhemoglobin to methemoglobin, which proceeds spectral changes may be understood in terms of a transition 630 nm, consistent with that of native methemoglobin. These resulting spectral profile showed a maximum absorbance at 5

The Pronase-digested hemoglobin was chromatographed on a Bondapak C-18 column (5 mm, 30 cm) using a mobile phase 30 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 mg/ml) was treated with mushroom tyrosinase or polyphenol oxidase (10 U/ml) and incubated for 30 min at 37 °C. After 30 min at 37 °C, the samples were centrifuged in microcentrifuge. 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 under “Materials and Methods.” A, the pH profile was carried out with 0.2 mM sodium phosphate (pH 5–8) or 0.2 mM HEPES buffer (pH 8–11). The fluorescence was measured in an Aminco-Bowman spectrophotometer with quinine sulfate as a standard, at the following pairs of wavelengths: \( \lambda_{	ext{excitation}} = 283 \text{nm} \) and \( \lambda_{	ext{emission}} = 315 \text{nm} \) (filled circles) and \( \lambda_{	ext{excitation}} = 315 \text{nm} \) and \( \lambda_{	ext{emission}} = 425 \text{nm} \) (open circles). B, as described for A, but using 0.1 mM pure tyrosine. Inset, emission spectra of PCA-neutralized supernatant (A) or pure tyrosine (B) using \( \lambda_{	ext{excitation}} = 283 \text{nm} \) at pH 5 (solid line) or pH 7.4 (dotted line).

**Table I**

Tyrosine oxidation products in hydrogen peroxide-treated hemoglobin

| Pronase-digested samples | Tyrosine oxidation products (peak height ratio of product/tyrosine) |
|--------------------------|---------------------------------------------------------------|
|                          | Dopamine quinone (1.34 min) | Dopamine (3.27 min) | Dihydroxyindol (7.38 min) | Dityrosine (5.22 min) |
| Hemoglobin fragments     | 1.07                        | 0.60               | 1.38                       | 0.92               |
| Hemoglobin               | 3.22                        | 4.84               | 1.46                       |                   |

Effect a hydrogen abstraction, although the latter may be less efficient than hydroxyl radical because of a restricted steric hindrance), and (iii) a protein-centered radical.

A role for ferrylhemoglobin, as the oxidant species during the oxidation of oxyhemoglobin by a continuous flux of H2O2, is substantiated by the following experimental results: (i) a transient visible spectrum ascribed to ferrylhemoglobin (Fig. 5A), (ii) an EPR spectrum of the protein radical (Fig. 5B), and (iii) the lack of a 5,5'-dimethyl-1-pyrroline-N-oxide-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 H2O2/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 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-FeIV-OH and X-FeV-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 H2O2 (4, 5) and with ribonucleoside diposphosphate reductase (29), was detected by mixing oxyhemoglobin and H2O2 (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 H2O2/min), and the spin trap 5,5'-dimethyl-1-pyrroline-N-oxide at concentrations of 80–200 μM (data not shown). Although hydroxyl radical formation from hemoglobin-catalyzed homolytic cleavage of H2O2 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 H2O2-treated Oxyhemoglobin**—It has been reported that the protein radical formed in ferrylmyoglobin could decay (i) by tyrosine addition to the heme group, as described by Catalano et al. (20) for myoglobin, (ii) by dityrosine cross-link formation as in sperm whale myoglobin (19), or (iii) by an additional oxidation of the aromatic radical in a close proximity to the heme group 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-dioxyn complex (HX-FeII-O-O

radical) 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-dioxyn complex). The formation of Tyr oxidation products might involve cyclization, decarboxylation, and further oxidation steps on either the apoprotein or fragments released from the protein. The chemistry of the reaction between hemoglobin and H2O2 is complicated not only because several oxidizing species might contribute to the formation of oxidized products, but also because 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

and by others (36, 37) seem to rule out this possibility, and an iron-dioxyn complex (HX-FeII-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-dioxyn complex). The formation of Tyr oxidation products might involve cyclization, decarboxylation, and further oxidation steps on either the apoprotein or fragments released from the protein. The chemistry of the reaction between hemoglobin and H2O2 is complicated not only because several oxidizing species might contribute to the formation of oxidized products, but also because 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

and by others (36, 37) seem to rule out this possibility, and an iron-dioxyn complex (HX-FeII-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-dioxyn complex). The formation of Tyr oxidation products might involve cyclization, decarboxylation, and further oxidation steps on either the apoprotein or fragments released from the protein. The chemistry of the reaction between hemoglobin and H2O2 is complicated not only because several oxidizing species might contribute to the formation of oxidized products, but also because 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.
above can be explained by the participation of either one or two monomers of hemoglobin (α or β subunit), whereas 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 H₂O₂ Flux*—Bovine, rabbit, or human intact RBC (5%, v/v) exposed to different flow rates of H₂O₂ 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 minimal (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 H₂O₂ can be explained by (i) a peroxidatic activity of ferrylhemoglobin using tyrosine (from the intracellular pool of amino acids) as a substrate or (ii) proteolytic release of dityrosine previously formed in the oxidatively modified protein.

When a reaction mixture of 0.2 mM oxyhemoglobin and 1.7 mM H₂O₂ was supplemented with 25 mM l-tyrosine 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 six times (per heme) more efficient than hemoglobin in catalyzing the same reaction (Fig. 9A, inset).

Lysed RBC incubated with H₂O₂ 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). Because catalase concentration increased with increasing concentration of protein additions, the experiment was repeated in the presence of 0.1 mM 3-amino-1,2,4-triazole (aminotriazole), an inhibitor of the catalase-hydrogen peroxide Complex I through the binding of aminotriazole to a His residue in the peptide VVHAK (43); the target sequence is highly conserved among catalases from different species, but it is not present in hemoglobin or myoglobin.

The results shown in Fig. 9B indicate that RBC experiencing a flux of H₂O₂ 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 × 10⁻⁴ M (Fig. 9B, inset), a concentration eight times higher than the actual intracellular concentration of free tyrosine, corrected by the volume of water excluded by hemoglobin (60–70 μM).² 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: (i) to characterize the oxidation products formed after exposing oxyhemoglobin to a flux of H₂O₂, (ii) to provide a plausible mechanism for the formation of these products, and (iii) to test the feasibility of dityrosine measurements as markers for oxidative stress and for selective proteolysis.

² Given the volume of a spherical molecule (59 m³) and the radius of Hb (r = 2.75 × 10⁻¹¹ cm; Ref. 44), the volume per molecule can be calculated as 8.7 × 10⁻¹⁰ cm³. Considering that [Hb] in RBC is 34 g/100 ml, Mₚ = 64,500, and the average volume of RBC for an adult man is 87 μm³ (44, 45), then the volume for unsolvated Hb molecules contained in a RBC is 2.4 × 10⁻¹³ cm³. This indicates that unsolvated Hb occupies 27.8% of the total volume of RBC. The partial specific volume for Hb can be estimated to be close to that of myoglobin (0.741 cm³/g; Ref. 46), giving the homologies between these two proteins. Thus, the partial specific volume of Hb (νₚ) plus the specific volume of water (νₚ = 1 cm³/g) times the amount of hydration (δ) can be calculated from the following equation: (νₚ + δ) = νₛ = νₛ + δ νₛ, where νₛ = 1.105 (46). The volume of solvated Hb is 0.999 cm³/g, representing 34% of the total RBC volume (0.999 cm³/g × 2.96 × 10⁻¹³ g of Hb). If we correct the concentration of free tyrosine in adult RBC (mean, 42 μM; Ref. 39) considering the free water, then the concentration would be equal to 64 μM, about eight times lower than the threshold for dityrosine production.
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 that involved cyclization, hydrogen 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 bond is formed, it is resistant to hydrolysis by all other lytic enzymes in the red cell (21). Dityrosine can also be measured by HPLC with (48) or without (this paper and Ref. 49) derivatization or by high efficiency thin layer chromatography, 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, as has been demonstrated by Heinke 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.

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 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; Ref. 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

1. Caughey, W. S., and Watkins, J. A. (1985) Handbook of Methods for Oxygen Radical Research (Greenwald, R. A., ed) pp. 95–104, CRC Press, Inc., Boca Raton, FL
2. Giulivi, C., Hochstein, P., and Davies, K. J. A. (1994) Free Radic. Biol. Med. 16, 123–129
3. Boveris, A., Chance, B., and Sies, H. (1979) Physiol. Rev. 59, 527–605
4. Grune, T., and Davies, K. J. A. (1997) FASEB J. 11, 536–534
5. Giulivi, C., Romero, J. F., and Cadenas, E. (1992) Arch. Biochem. Biophys. 299, 302–312
6. Giulivi, C., and Cadenas, E. (1993) Arch. Biochem. Biophys. 303, 152–158
7. Giulivi, C., and Cadenas, E. (1993) FEBS Lett. 332, 287–290
8. Galaris, D., Cadenas, E., and Hochstein, P. (1989) Arch. Biochem. Biophys. 273, 497–504
9. Galaris, D., Mira, D., Sevanian, A., Cadenas, E., and Hochstein, P. (1988) Arch. Biochem. Biophys. 262, 221–231
10. Giulivi, C., and Cadenas, E. (1998) Free Radic. Biol. Med. 25, 175–183
11. Bruckdorfer, K. R.; Rice-Evans, C., and Dee, G. (1990) Biochem. Soc. Trans. 18, 1061–1063
12. Kanner, J., and Harel, S. (1985) Lipids 20, 625–628
13. Kanner, J., and Harel, S. (1985) Arch. Biochem. Biophys. 237, 314–321
14. Gibson, J. F., Ingram, D. E., and Nichols, P. (1958) Nature 181, 1398–1399
15. King, N. K., Looney, F. D., and Winfield, M. E. (1967) Biochim. Biophys. Acta 133, 65–82
16. Yonetani, T., and Schleyer, H. (1967) J. Biol. Chem. 242, 1974–1979
17. Harada, K., and Yamazaki, I. (1987) J. Biochem. (Tokyo) 101, 283–286
18. Miki, H., Harada, K., Yamazaki, I., Tamura, M., and Watanabe, H. (1989) Arch. Biochem. Biophys. 275, 354–362
19. Tew, D., and Ortiz de Montellano, P. R. (1989) J. Biol. Chem. 263, 17880–17886
20. Catalano, C. E., Choe, Y. S., and Ortiz de Montellano, P. R. (1989) J. Biol. Chem. 264, 10534–10541
21. Giulivi, C., and Davies, K. J. A. (1993) J. Biol. Chem. 268, 8752–8759
22. Di Iorio, E. E. (1981) Methods Enzymol. 76, 57–87
23. Gross, A. J., and Sizer, I. W. (1959) J. Biol. Chem. 234, 1611–1614
24. Giulivi, C., and Davies, K. J. A. (1990) J. Biol. Chem. 265, 19453–19460
25. Boveris, A., Martino, E., and Stoppani, A. O. M. (1977) Anal. Biochem. 80, 145–158
26. Leonard, L. J., Townsend, D., and King, R. A. (1988) Biochemistry 27, 6156–6159
27. Segan-Aguilar, J., and Lind, C. (1989) Chem. Biol. Interact. 72, 309–324
28. Shiga, T., and Imazumi, K. (1985) Arch. Biochem. Biophys. 187, 469–479
29. Reichard, P., and Ehrenberg, A. (1983) *Science* **221**, 514–516
30. Sadrzadeh, S. M. H., Graf, E., Panter, S. S., Hallaway, P. E., and Eaton, J. W. (1984) *J. Biol. Chem.* **259**, 14354–14356
31. Gutteridge, J. M. C. (1986) *FEBS Lett.* **201**, 291–295
32. Prutz, W. A., Butler, J., and Land, E. J. (1983) *Int. J. Radiat. Biol.* **44**, 183–196
33. Cudina, I., and Josimovic, L. (1987) *Radiat. Res.* **109**, 206–215
34. Ortiz de Montellano, P. R., and Catalano, C. E. (1986) *J. Biol. Chem.* **260**, 9265–9271
35. Allentoff, A. J., Bolton, J. L., Wils, A., Thompson, J. A., and Ortiz de Montellano, P. R. (1992) *J. Am. Chem. Soc.* **114**, 9744–9749
36. Hunter, E. P., Desroisiers, M. F., and Simic, M. G. (1989) *Free Radic. Biol. Med.* **6**, 581–585
37. Kelman, D. J., and Mason, R. P. (1992) *Free Radic. Res. Commun.* **16**, 27–33
38. Giulivi, C., and Cadenas, E. (1998) *Free Radic. Biol. Med.* **24**, 269–279
39. Soupart, P. (1962) *Amino Acid Pools* (Holden, J. T., ed) p. 263, Elsevier Science Publishers B.V., Amsterdam
40. Margoliash, E., Novogrodsky, A., and Schejter, A. (1960) *Biochem. J.* **74**, 339–348
41. Tephly, T. R., Mannerling, G. J., and Parks, R. E., Jr. (1961) *J. Pharmacol. Exp. Ther.* **134**, 77–82
42. Nicholls, P. (1962) *Biochim. Biophys. Acta* **59**, 414–420
43. Chang, Y., and Schroeder, W. A. (1972) *Arch. Biochem. Biophys.* **148**, 505–508
44. Stryer, L. (1981) *Biochemistry*, 2nd Ed., pp. 59–64, W. H. Freeman and Company, San Francisco, CA
45. Diem, K., and Lentner, C., eds (1974) *Scientific Tables*, pp. 574, 613, Geigy Pharmaceuticals, Division of Ciba-Geigy Corporation, Ardsley, NY
46. Tinoco, J. I., Sauer, K., and Wang, Y. C. (1978) *Physical Chemistry: Principles and Applications in Biological Sciences*, 3rd Ed., pp. 282, 308, Prentice Hall, Englewood Cliffs, NJ
47. Giulivi, C., Pacifici, R. E., and Davies, K. J. A. (1994) *Arch. Biochem. Biophys.* **311**, 329–341
48. Malencik, D. A., and Anderson, S. R. (1987) *Biochemistry* **26**, 695–704
49. Malencik, D. A., Sprouse, J. F., Swanson, C. A., and Anderson, S. R. (1996) *Anal. Biochem.* **242**, 202–213
50. Leeuwenburgh, C., Rasmussen, J. E., Hsu, F. F., Mueller, D. M., Pennathur, S., and Heinecke, J. W. (1997) *J. Biol. Chem.* **272**, 3520–3526
51. Heinecke, J. W. (1999) *FASEB J.* **13**, 1113–1120
52. Giulivi, C., and Davies, K. J. A. (1994) *Methods Enzymol.* **233**, 363–371
53. Stadtman, E. R. (1993) *Annu. Rev. Biochem.* **62**, 797–821
54. Levine, R. L., Williams, J. A., Stadtman, E. R., and Shacter, E. (1994) *Methods Enzymol.* **233**, 346–357