A Novel Antioxidant Role for Hemoglobin

THE COMPROPORTIONATION OF FERRYLHEMOGLOBIN WITH OXYHEMOGLOBIN*

Cecilia Giulivić and Kelvin J. A. Davies$

From the Institute for Toxicology and the Department of Biochemistry, University of Southern California, Health Sciences Campus, Los Angeles, California 90033

Ferrylhemoglobin (X-Fe$^IV$-OH, where X denotes an amino acid residue in the globin moiety) has long been suspected as a cytotoxic agent produced by the interaction of oxyhemoglobin (X-Fe$^III$O$_2$) or methemoglobin (X-Fe$^III$) with H$_2$O$_2$ in red blood cells. To date, however, technical difficulties have prevented the identification and quantification of X-Fe$^IV$-OH. Oxyhemoglobin exposed to a continuous flux of H$_2$O$_2$ (generated at a rate of 120 μM/min during the glucose oxidase-catalyzed oxidation of glucose) was oxidized to (a) X-Fe$^IV$-OH when [X-Fe$^III$O$_2$] < 75 μM and (b) X-Fe$^III$ when [X-Fe$^III$O$_2$] > 75 μM (the production of X-Fe$^IV$ proceeded with intermediate formation of X-Fe$^{IV}$-OH). The reduction of the X-Fe$^IV$ OH to X-Fe$^{III}$ could be explained by either of two alternative mechanisms: a O$_2$-mediated X-Fe$^IV$-OH → X-Fe$^{III}$ transition or a comproportionation of X-Fe$^{IV}$-OH and X-Fe$^{III}$O$_2$ to yield X-Fe$^{III}$ (a process mediated by a tyrosine moiety in the hemoprotein). The low rate of X-Fe$^{III}$O$_2$ autodissociation plus the negligible decrease in the rate of X-Fe$^{III}$ formation in the presence of either native or heat-denatured superoxide dismutase or apoenzyme (1 μM) suggested that O$_2$ does not contribute to the reduction of X-Fe$^IV$-OH. Moreover, the dependence of X-Fe$^{III}$ formation on X-Fe$^{III}$O$_2$ concentration, together with the results of O$_2$ uptake and H$_2$O$_2$ consumption measurements, provide experimental evidence to support the comproportionation reaction. Comproportionation is apparently catalyzed by intermolecular electron transfer between tyrosine residues, since the reaction did not occur when tyrosine residues were blocked by acetylation. Intact red blood cells exposed to the same flow rate of H$_2$O$_2$ presented a spectral profile which could be explained as a transition from X-Fe$^{III}$O$_2$ to X-Fe$^{IV}$-$^{III}$. The intermediate production of X-Fe$^{IV}$-OH was detected by adding Na$_2$S (2 mM), which revealed a spectral profile identical with that obtained with purified X-Fe$^IV$-OH. Measurements of concentrations and relative rate constants for the reaction of various intracellular reductants (glutathione, NAD(P)H, uric acid, ascorbic acid) with X-Fe$^{IV}$-OH revealed that comproportionation of X-Fe$^{IV}$-OH with X-Fe$^{III}$O$_2$ is the favored reaction. Our results provide (to our knowledge) the first definitive evidence for X-Fe$^{IV}$-OH in intact red blood cells. The rapid comproportionation reaction between X-Fe$^{IV}$-OH and X-Fe$^{III}$O$_2$ (to produce X-Fe$^{III}$) explains why X-Fe$^{IV}$-OH has been elusive to date. Moreover, the comproportionation reaction represents an important and novel antioxidant function for red blood cell X-Fe$^{III}$O$_2$, in which the cytotoxic X-Fe$^{IV}$-OH is quenched and the resultant X-Fe$^{III}$ is re-reduced by methemoglobin reductase.

Hemoglobin (Hb), the major heme protein of red blood cells, is responsible for the transport of O$_2$ to the tissues. This function depends upon the ability of the ferrous iron in the heme to bind and release O$_2$ (1). Oxyhemoglobin (X-Fe$^III$O$_2$, where X denotes an amino acid residue in the globin molecule) autoxidizes at a relatively slow rate to yield methemoglobin (X-Fe$^{III}$) and superoxide anion (O$_2^-$) (2–4), which dismutates to hydrogen peroxide (H$_2$O$_2$). Current interest in the mechanisms by which reactive oxygen species can cause cellular injury in several tissues has focused on the potential cytotoxicity of hemoprotein/H$_2$O$_2$ interactions (5–9). The oxidation of the heme iron in X-Fe$^{III}$O$_2$ 1 and 2 oxidizing equivalents above Fe$^II$ results in the formation of X-Fe$^{III}$ and ferrylhemoglobin (X-Fe$^{IV}$-OH), respectively (10). Although the initial reactions between X-Fe$^{III}$O$_2$ and H$_2$O$_2$ are complicated by subsequent interactions of the products with H$_2$O$_2$, it is clear that X-Fe$^{IV}$-OH is a strong oxidant which can promote oxidation (11), peroxidation (5), and epoxidation (12) of various biomolecules in vitro. Prolonged reaction of Hb with H$_2$O$_2$ also leads to Hb damage, both in vitro and in vivo. Indeed, Hb that has been oxidatively denatured by H$_2$O$_2$ is recognized and selectively degraded in both erythrocytes and reticuloocytes (13–16) by a 670-kDa protease complex for which we have suggested the trivial name macroxyproteinase (17). Although the ferryl complex in X-Fe$^{IV}$-OH has all the attributes necessary to be considered an important species in free radical damage processes, the generation of this compound in red blood cells has yet to be demonstrated (11).

In the present work, we have used purified Hb and intact erythrocytes to study the reaction of X-Fe$^{III}$O$_2$ with H$_2$O$_2$. Hydrogen peroxide was produced in a continuous flow in order to best mimic cellular conditions. The results presented here describe the mechanism of formation of X-Fe$^{IV}$-OH, from the initial reaction of H$_2$O$_2$ with X-Fe$^{III}$O$_2$ to the subsequent fate of X-Fe$^{IV}$-OH.

1 The abbreviations used are: Hb, hemoglobin; X-Fe$^{III}$O$_2$, oxyhemoglobin, where X denotes an amino acid residue in the globin molecule; X-Fe$^{IV}$, methemoglobin; X-Fe$^{IV}$-OH, oxoferryl compound or ferrylhemoglobin; RBC, red blood cells.
MATERIALS AND METHODS

Chemicals, Biochemicals, and Biological Materials—X-Fe\textsuperscript{II}O\textsubscript{2} was prepared daily from 2 \times crystalized bovine X-Fe\textsuperscript{III} (Sigma, product H-2500) or human X-Fe\textsuperscript{III} (Sigma, product H-7379) by reduction with dithionite (molar ratio, 1:2), in deaerated 0.01 M potassium phosphate buffer (pH 7.8). Reduced Hb was chromatographically purified on a Sephadex G-25 column (30 \times 1.5 cm) using 0.01 M potassium phosphate buffer (pH 7.8) as an eluant (18). A small percentage of X-Fe\textsuperscript{III} (2–3%) was always present in the solutions of X-Fe\textsuperscript{II}O\textsubscript{2} so obtained, due to an unavoidable autoxidation. The chromatographed X-Fe\textsuperscript{II}O\textsubscript{2} was concentrated in Centricom™ microconcentrator tubes (30-kDa cutoff; Amicon Division, W. R. Grace & Co.). Fresh human, bovine, and rabbit RBC were obtained as previously described (14). All other reagents were of analytical grade.

Hydrogen Peroxide Exposure—Glucose oxidase from Aspergillus niger (A grade, Calbiochem, product 346355), which catalyzes the reaction glucose + O\textsubscript{2} \rightarrow gluconic acid + H\textsubscript{2}O\textsubscript{2}, was added to cuvettes containing either bovine X-Fe\textsuperscript{II}O\textsubscript{2} or intact RBC in Krebs-Ringer phosphate buffer (pH 7.4, 25 °C) plus 5 mM glucose for 30 min. The glucose oxidase activity was determined as described by Boveris et al. (19).

Spectrophotometric Assays—Optical absorption spectra were recorded in a dual-wavelength, double-beam spectrophotometer (Shimadzu Corp., Japan, model UV-2000). X-Fe\textsuperscript{III} formation was followed at 630 nm (20), and H\textsubscript{2}O\textsubscript{2} consumption was measured at 240 nm (ε = 40 M\textsuperscript{-1} cm\textsuperscript{-1}). H\textsubscript{2}O\textsubscript{2} concentrations were determined spectrofluorometrically by the formation of a fluorescent dimer of \textit{n}-hydroxyphenylacetic acid (310 nm excitation, 415 nm emission) during the reaction catalyzed by horseradish peroxidase, as described by Danner et al. (21) and Hinsberg et al. (22).

Oxygen Uptake—The O\textsubscript{2} consumption of glucose + glucose oxidase, plus or minus X-Fe\textsuperscript{II}O\textsubscript{2}, was measured with a Gilson model 5/6 H \textit{oxigraph} (Gilson Medical Electronics, Inc., Middleton, WI) fitted with a Clark-type O\textsubscript{2} electrode (Rank Brothers, Bottisham, United Kingdom). The reaction mixtures were prepared as described in the spectrometric assay section above.

RESULTS AND DISCUSSION

Spectrophotometric Analysis of X-Fe\textsuperscript{II}O\textsubscript{2} Oxidation by H\textsubscript{2}O\textsubscript{2}—H\textsubscript{2}O\textsubscript{2}, generated at a rate of 120 μM/min during the oxidation of glucose by glucose oxidase, exerted in the absorption spectrum of 20 μM X-Fe\textsuperscript{II}O\textsubscript{2} a decrease in the absorbance at 540 and 577 nm accompanied by the occurrence of two new peaks at 545 and 580 nm (Fig. 1). The increase in absorption at 545 and 580 nm might indicate a compound and H\textsubscript{2}O (Equation 2). In the presence of a high concentration of X-Fe\textsuperscript{II}O\textsubscript{2} (i.e. 10 times higher), an additional peak at 630 nm was observed (Fig. 2). The increase in absorbance at 630 nm is consistent with the formation of X-Fe\textsuperscript{III}O\textsubscript{2} (Fig. 2). The spectral changes of Figs. 1 and 2 could be explained by a transition from X-Fe\textsuperscript{II}O\textsubscript{2} to X-Fe\textsuperscript{III}, with the intermediate formation of X-Fe\textsuperscript{IV}O\textsubscript{H}.

The initial oxidation of X-Fe\textsuperscript{II}O\textsubscript{2} by H\textsubscript{2}O\textsubscript{2} can be described in terms of Equation 1, which yields X-Fe\textsuperscript{II}O\textsubscript{2} and the ferryl compound (X-Fe\textsuperscript{IV}O\textsubscript{H}).

\[ \text{H}^+ + \text{X-Fe}^{\text{II}O_2} + \text{H}_2\text{O}_2 \rightarrow \text{X-Fe}^{\text{IV}O_H} + \text{O}_2 + \text{H}_2\text{O} \]  

(1)

The oxidation of X-Fe\textsuperscript{II}O\textsubscript{2} by H\textsubscript{2}O\textsubscript{2} could occur by an alternative pathway that might proceed with Equation 2 as the first step (23, 24). Equation 2, which is reminiscent of a Fenton-like reaction, implies the intermediacy of X-Fe\textsuperscript{III} in the oxidation of Fe\textsuperscript{II} to Fe\textsuperscript{III}.

\[ \text{X-Fe}^{\text{II}O_2} + \text{H}_2\text{O}_2 \rightarrow \text{X-Fe}^{\text{II}I} + \text{HO}^+ + \text{OH}^- + \text{O}_2 \]  

(2)

Equation 2, which suggests Hb as a Fenton reagent (25), has been previously discarded (26, 27). In the solution of X-Fe\textsuperscript{III}O\textsubscript{H} Fenton-like reactions was reported as the interaction of iron or low molecular weight iron complexes with H\textsubscript{2}O\textsubscript{2} and an excess of H\textsubscript{2}O\textsubscript{2} is a required condition to release iron from the heme (26, 27). Furthermore, the absence of an intermediate X-Fe\textsuperscript{III} spectrum in Fig. 1 suggests that Equation 1 should be considered a more likely explanation for the initial oxidation of X-Fe\textsuperscript{II}O\textsubscript{2} by peroxide.

Spectral changes that could be ascribed to X-Fe\textsuperscript{III} as a final product (Fig. 2) and which were revealed by oxidation of Hb by H\textsubscript{2}O\textsubscript{2} at high concentrations of X-Fe\textsuperscript{II}O\textsubscript{2} could be explained if the following reactions had taken place (Equations 3–5).

\[ \text{X-Fe}^{\text{III}} - \text{OH} + \text{H}_2\text{O}_2 \rightarrow \text{X-Fe}^{\text{II}I} + \text{H}_2\text{O} + \text{H}_2\text{O}_2 \]  

(3)

\[ \text{X-Fe}^{\text{II}I} + \text{H}_2\text{O}_2 + \text{H}^+ \rightarrow \text{H}_2\text{O} + \text{X-Fe}^{\text{IV}-\text{OH}} \]  

(4)

\[ \text{X-Fe}^{\text{IV}-\text{OH}} + \text{X-Fe}^{\text{II}O_2} \rightarrow 2\text{X-Fe}^{\text{II}I} + \text{O}_2 \]  

(5)

In our experimental model, involving a continuous flow of H\textsubscript{2}O\textsubscript{2}, it could be expected that the peroxide would react further with X-Fe\textsuperscript{IV}O\textsubscript{H}, yielding X-Fe\textsuperscript{II} and hydroxyl radical (HO\textsubscript{2}, Equation 3). In the presence of low concentrations of X-Fe\textsuperscript{II}O\textsubscript{2}, the rate of H\textsubscript{2}O\textsubscript{2} generation should be high enough to react with any X-Fe\textsuperscript{III} formed, yielding the ferryl compound and H\textsubscript{2}O (Equation 4). In the presence of a high concentration of hemoprotein, however, most of the H\textsubscript{2}O\textsubscript{2} should be rapidly consumed in Equation 1, and Reaction 4 would not be favored. Under such conditions, a comprop-
the equilibria of Reactions 1 and 5 would be displaced toward the concentration of $\text{X-Fe}^{II}_0$ increases above 75 concentrations below 75.

Harada et al. (28) have provided indirect evidence for a similar reaction between $\text{X-Fe}^{II}_0$ and $\text{X-Fe}^{IV}_0$-OH, yielding products after the addition of glucose oxidase. $\text{X-Fe}^{II}_0$ was used. Repetitive scans were recorded at 10-min intervals from the data presented so far can be described as electron transfer mediated by $\text{O}_2$. These reactions would have to involve an initial autooxidation of $\text{X-Fe}^{II}_0$ to generate $\text{O}_2$ and $\text{X-Fe}^{III}$ ($\text{X-Fe}^{II}_0 \rightarrow \text{X-Fe}^{III} + \text{O}_2 + \text{H}_2\text{O}$). $\text{X-Fe}^{III}$ could then react further with $\text{H}_2\text{O}_2$ by either a homolytic or a heterolytic scission (10). The former implies the transient formation of $\text{H}_2\text{O}_2$ and to the surrounding medium (12), facilitates the formation of the protein radical form of $\text{X-Fe}^{IV}_0$-OH. Therefore, the net balance obtained from the homolytic scission would be the same as in the heterolytic scission, $\text{H}_2\text{O}_2$ and $\text{X-Fe}^{IV}_0$-OH. The $\text{O}_2$ resulting from $\text{Hb}$ autooxidation might reduce $\text{X-Fe}^{IV}_0$-OH to $\text{X-Fe}^{III}$ with the release of $\text{O}_2$. ($\text{H}^+ + \text{X-Fe}^{IV}_0$-OH + $\text{O}_2 \rightarrow \text{X-Fe}^{III} + \text{O}_2 + \text{H}_2\text{O}$) or might slowly dismutate to $\text{H}_2\text{O}_2$ and $\text{O}_2$ ($\text{O}_2^+ + 2\text{H}^+ \rightarrow \text{H}_2\text{O}_2 + \text{O}_2$).

Although the balance of such reactions would be indistinguishable from that of Equation 5, both mechanisms can be evaluated in terms of their relative rates of production of $\text{X-Fe}^{III}$. Importantly, the autooxidation of $\text{X-Fe}^{II}_0$ has been

**FIG. 2. Oxidation of $\text{X-Fe}^{II}_0$ to $\text{X-Fe}^{III}$ by $\text{H}_2\text{O}_2$.** This experiment was an exact repeat of that in Fig. 1 except that 200 $\mu$M bovine $\text{X-Fe}^{II}_0$ was used. Repetitive scans were recorded at 10-min intervals after the addition of glucose oxidase.

**Equation 5** constitutes a likely explanation for the results of Fig. 2, since (in those experiments) the concentration of $\text{X-Fe}^{II}_0$ was high enough to react with any $\text{X-Fe}^{IV}_0$-OH formed. Furthermore, Equation 5 predicts that $\text{X-Fe}^{III}$ formation should be directly related to $\text{X-Fe}^{II}_0$ concentration, and this prediction can be explored experimentally. To test this hypothesis, we exposed different concentrations of bovine and human $\text{X-Fe}^{II}_0$ to a continuous flux of $\text{H}_2\text{O}_2$ and measured the formation of $\text{X-Fe}^{III}$ and $\text{X-Fe}^{IV}_0$-OH (Fig. 3). Significant $\text{X-Fe}^{IV}_0$-OH formation was observed at $\text{X-Fe}^{II}_0$ concentrations below 75 $\mu$M, whereas above this concentration, $\text{X-Fe}^{III}$ formation predominated. The lack of $\text{X-Fe}^{III}$ formation at concentrations of $\text{X-Fe}^{II}_0$ below 75 $\mu$M (Fig. 3) can be explained if the contribution of Reaction 5 is negligible. As the concentration of $\text{X-Fe}^{II}_0$ increases above 75 $\mu$M, however, the equilibria of Reactions 1 and 5 would be displaced toward the right, with the formation of the ferryl compound and $\text{X-Fe}^{III}$.
Fig. 3. Dependence of \( X-Fe^{IV}\)-OH (●) and \( X-Fe^{III}\) (▲) formation upon the initial concentration of bovine (panel A) or human \( X-Fe^{IV}\)O₂ (panel B). \( X-Fe^{III}\) and \( X-Fe^{IV}\)-OH formation was recorded by repetitive spectrophotometric scans after the addition of glucose oxidase (7.5 μg/ml) to various concentrations of \( X-Fe^{IV}\)O₂, as described in the legends to Figs. 1 and 2. \( X-Fe^{III}\) was measured according to Winterbourn (20) and \( X-Fe^{IV}\)-OH as the difference between total Hb minus \( X-Fe^{III}\).

![Graph showing concentration vs. \( X-Fe^{III}\) and \( X-Fe^{IV}\)-OH formation](image)

Fig. 4. Inhibition of the comproportionation reaction between \( X-Fe^{III}-OH \) and \( X-Fe^{IV}O_2 \) by the \( \alpha \)-acetylation of hemoglobin tyrosine residues by \( N \)-acetylimidazole. Acetylation of tyrosine hydroxyl groups of hemoglobin with \( N \)-acetylimidazole was carried out in 50 mM Tris-HCl buffer (pH 7.4) at 25 °C according to Janig et al. (45). The reactions were initiated by addition of solid \( N \)-acetylimidazole, in a molar excess from 0 to 2000, to a volume of 1 ml of 20 μM bovine hemoglobin. After 15 min of incubation, the excess \( N \)-acetylimidazole and its reaction product, imidazole, were removed by chromatography on a Sephadex G-25 column (30 × 1 cm) using 0.1 M potassium phosphate buffer (pH 7.4) for column equilibration and elution. The number of acetylated tyrosine moieties per molecule of hemoglobin was calculated by measuring the decrease in 278 nm absorption and using 1.16 mM⁻¹ cm⁻¹ as the difference absorption coefficient for \( \alpha \)-acetylintyrosine residues, resulting from the decrease of tyrosine absorption from 1.23 to 0.07 mM⁻¹ cm⁻¹. The modified and control proteins were concentrated by centrifuging in Centricom tubes (molecular mass cutoff of 10 kDa). Control and acetylated hemoglobins (20 μM) were treated with \( H_2O_2 \) in a molar ratio of 5:1. Catalase (0.1 μM) was then added to the reaction mixture to decompose excess \( H_2O_2 \). Oxymyoglobin (prepared as described under “Materials and Methods”) was added to the cuvette at various concentrations. The rate of comproportionation was measured as the increase in the absorbance at 650 nm (control rate \( \Delta A_{650} = 0.0125/\text{min} \)).

reported to occur at a very low rate (1–3% of Hb is oxidized daily in the RBC) (35–37) that cannot account for the rate of \( X-Fe^{III} \) obtained in our experimental system within 30 min. Moreover, in the presence of superoxide dismutase \( O_2^- \) consumption by \( X-Fe^{IV}-OH \) should be almost completely inhibited, whereas Reaction 5 would not be affected by the enzyme. In the presence of superoxide dismutase, a decrease in the rate of \( X-Fe^{III} \) formation could be expected if the reduction of \( X-Fe^{IV}-OH \) by \( O_2^- \) was of quantitative significance. Since the rate of \( X-Fe^{IV} \) formation was unchanged by the addition of 1 μM native superoxide dismutase, heat-denatured superoxide dismutase, or the apoenzyme (data not shown), we conclude that superoxide-mediated reduction of \( X-Fe^{IV}-OH \) is a reaction of minor relevance in the formation of \( X-Fe^{III} \), i.e. we suggest that the tyrosine-dependent comproportionation reaction (Equation 5) is the favored mechanism for \( X-Fe^{III} \) production.

The oxidation of \( X-Fe^{IV}O_2 \) consumed nonstoichiometric amounts of \( H_2O_2 \). The \( H_2O_2 \) production by the glucose + glucose oxidase system is shown in Fig. 5. The initial rate was found to be 120 μM/min, which was in agreement with measurements of \( O_2 \) uptake (data not shown). The maximal rate was achieved 1.5 min after starting the reaction. The addition of \( X-Fe^{III}O_2 \) (140 μM) significantly decreased the \( H_2O_2 \) concentration, suggesting that \( H_2O_2 \) was consumed by the hemoprotein (Fig. 5). From these data, we obtained both the \( H_2O_2 \) consumed by \( X-Fe^{III}O_2 \) during the 30 min of incubation and the initial rate of \( H_2O_2 \) consumption, 90 μM/min (Fig. 5, inset).

The experimental results obtained with concentrations of \( X-Fe^{III}O_2 \) above 25 μM showed no further oxidation of \( X-Fe^{III} \) (formed in Equation 5 by \( H_2O_2 \), although there was a continuous flux of \( H_2O \) generated by the glucose + glucose oxidase system (Fig. 2). This can be understood if the remaining concentration of \( H_2O_2 \) was not high enough to react with the \( X-Fe^{III} \) formed in our reaction mixture. This explanation seemed to agree with the results of \( H_2O_2 \) consumption by \( X-Fe^{III}O_2 \) shown in Fig. 5, which revealed that the remaining \( H_2O_2 \) (13–20 μM) was not enough to oxidize any \( X-Fe^{III} \) formed, as is demonstrated in Fig. 6. In Fig. 6, which shows the percentage of \( X-Fe^{IV}-OH \) formed at different ratios of \( X-Fe^{III} \) to \( H_2O_2 \), less than 5% of \( X-Fe^{III}-OH \) would be produced using an experimental ratio of \( H_2O_2/X-Fe^{III} \) of 0.1–0.2.

An alternative mechanism that might explain the \( H_2O_2 \) consumption of Fig. 5, inset, could involve a catalatic-like...
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Fig. 5. H$_2$O$_2$ production by the glucose + glucose oxidase system in the presence of X-Fe$^{IV}$O$_2$. The upper trace (,:) shows the concentration of H$_2$O$_2$ produced by the glucose + glucose oxidase system alone. The lower trace (7) shows H$_2$O$_2$ concentrations in the presence of glucose + glucose oxidase and bovine X-Fe$^{IV}$O$_2$. The assay conditions were as follows. The reaction mixture contained 5 mM glucose in Krebs-Ringer phosphate buffer (pH 7.4) and, where indicated, 70 $\mu$M X-Fe$^{III}$O$_2$ (oxy). The reaction was started by adding glucose oxidase (7.5 pg/ml) and aliquots were taken at the indicated times to measure remaining H$_2$O$_2$ by the horseradish peroxidase pH2O$_2$/H$_2$O$_2$ assay (19, 20). Inset, H$_2$O$_2$ consumed by X-Fe$^{IV}$O$_2$ during the oxidation of glucose by glucose oxidase. The results were obtained by subtracting the lower trace data from the upper trace data in the main part of the figure.

Fig. 6. X-Fe$^{IV}$-OH formation at different ratios of H$_2$O$_2$/Hb. The assay conditions were as follows. Bovine X-Fe$^{III}$ (50 $\mu$M) in Krebs-Ringer phosphate buffer (pH 7.4) was treated with different amounts of H$_2$O$_2$ to obtain X-Fe$^{IV}$-OH; the latter was followed spectrophotometrically at 556 nm. Inset, the double-reciprocal plot of the absorbance versus the ratio of H$_2$O$_2$/Hb exhibited a linear relationship (r = 0.98).

activity of the X-Fe$^{IV}$-OH. X-Fe$^{IV}$-OH has a characteristic absorption spectrum that resembles that of horseradish peroxidase compound II (36); indeed, both proteins share the same oxidation state (IV) of the heme iron (23, 24, 31). This similarity could explain the apparent peroxidatic activities of Hb in the presence of H$_2$O$_2$. In order to determine if X-Fe$^{IV}$-OH has a catalatic like activity, we measured spectrophotometrically the rate of H$_2$O$_2$ consumption by X-Fe$^{IV}$-OH in the presence of increasing concentrations of either peroxide (Fig. 7A) or the hemoprotein (Fig. 7B). The slopes of Fig. 7, A and B, which can be described as kA'[X-Fe$^{IV}$-OH] and kB'[H$_2$O$_2$], respectively, were used to calculate the rate constant between H$_2$O$_2$ and X-Fe$^{IV}$-OH, which was found to be 200 m$^{-1}$ s$^{-1}$. If the X-Fe$^{IV}$-OH were to exhibit any catalatic-like activity, the order in H$_2$O$_2$ should be 2, because two molecules of H$_2$O$_2$ would be decomposed by the ferryl compound in each cycle. In actuality, the logarithmic plot of the rate of H$_2$O$_2$ consumption against the concentration of either X-Fe$^{IV}$-OH or H$_2$O$_2$ (Fig. 7C) demonstrated that the order in H$_2$O$_2$ of the reaction was almost 1 (slopes = 0.96 and 0.98, respectively), thereby excluding a catalatic activity of the X-Fe$^{IV}$-OH.

Effect of X-Fe$^{IV}$O$_2$ on O$_2$ Evolution during the Glucose + Glucose Oxidase Reaction—Measurements of the rate of O$_2$ evolution as a function of X-Fe$^{IV}$O$_2$ concentration, in the presence of glucose oxidase, revealed that both were directly related (Fig. 8). The rate of O$_2$ evolution was found to be pseudo first-order in X-Fe$^{IV}$O$_2$ concentration with slopes of $1.4 \times 10^{-4}$ s$^{-1}$ and $3.5 \times 10^{-5}$ s$^{-1}$ at concentrations of X-Fe$^{IV}$O$_2$ below and above 20 $\mu$M, respectively (Fig. 8, inset).

In our experimental model, the main oxygen-consuming reaction is ascribed to the oxidation of glucose to gluconic acid.
acid by glucose oxidase. The rate of O₂ uptake may be described by Equation 6, where the single term corresponds to the oxidation of glucose by the FAD moiety of glucose oxidase.

In contrast, the rate of O₂ evolution may be described by Equation 7.

\[-d[O_2]/dt = k_1[H_2O_2][X-Fe^IV] + k_2[X-Fe^IV][X-Fe^V-OH] \]  
\[+ d[O_2]/dt = k_3[H_2O_2][X-Fe^III] + k_4[X-Fe^III][X-Fe^IV] \]  

Two main conclusions can be drawn from Fig. 8. (a) The first slope may be described as \(k_3[H_2O_2]\) because the contribution of Reaction 5 can be ignored at low concentrations of X-Fe⁴⁺. (b) As the hemoprotein concentration rises, the slope may be described as the addition of two terms, \(k_3[H_2O_2] + k_4[X-Fe^IV-OH]\). The decrease in the rate of O₂ evolution with increasing concentrations of X-Fe⁴⁺ is consistent with the observation that the H₂O₂ consumption decreases dramatically when high concentrations of X-Fe⁴⁺ are used (e.g. see Fig. 5). Under such conditions, therefore, the term \(k_3[H_2O_2]\) becomes negligible and may be ignored.

Mechanism of Hemoglobin Oxidation by H₂O₂—Taking into account the rates of H₂O₂ consumption and O₂ evolution, it is possible to propose a mechanism for the oxidation of X-Fe⁴⁺ by H₂O₂ (Scheme II). At concentrations of X-Fe⁴⁺ below 20 μM (i.e. 10 μM), the rate of O₂ evolution and H₂O₂ consumption can be represented by Equations 8 and 3, respectively.

\[+d[O_2]/dt = k_3[H_2O_2][X-Fe^III] = 45 \mu M/min \]  
\[-d[H_2O_2]/dt = k_4[X-Fe^IV][X-Fe^V-OH] + k_5[X-Fe^III][H_2O_2] \]  

If Reactions 3 and/or 4 were quantitatively significant, we would expect the rate of H₂O₂ consumption to be higher than the rate of O₂ release. In fact, the experimental data showed that the ratio of the rates of H₂O₂ consumed to O₂ released (at X-Fe⁴⁺ concentrations below 20 μM) was about 0.75 (Equation 9/Equation 8), indicating that Equations 3 and 4 are not operative. Our finding that the ratio of H₂O₂ consumed to O₂ released was below 1 indicates the possible significance of other H₂O₂-consuming reactions not previously considered.

One explanation involves the formation of a protein radical during the oxidation of X-Fe⁴⁺ by H₂O₂. Transient EPR-detectable protein radicals have indeed been observed in the outer protein surface, may react with molecular O₂ to yield a protein peroxy radical. Such O₂ incorporation may account for the experimental discrepancy found between the rates of H₂O₂ consumption and O₂ evolution.

On the other hand, at X-Fe⁴⁺ concentrations above 20 μM (i.e. 70 μM), the rates of O₂ evolution and H₂O₂ consumption may be described by Equations 7 and 10, respectively, where the former has a rate of 85 μM/min.

\[-d[H_2O_2]/dt = k_1[H_2O_2][X-Fe^IV] \]  
\[+ k_2[H_2O_2][X-Fe^III] = 90 \mu M/min \]  

The ratio between the rate of H₂O₂ consumed and O₂ evolved, at an X-Fe⁴⁺ concentration of 70 μM, was found to be 1.05, suggesting that Reactions 4 and 5 occur at comparable rates.

Spectral Analysis of X-Fe⁴⁺ Oxidation by H₂O₂ in Intact Red Blood Cells—It was of interest to study whether the proposed comproportionation mechanism would operate in intact RBC, which contain approximately 5 mM X-Fe⁴⁺. Fig. 9A shows the spectrum obtained by exposing intact human RBC to a continuous flow rate of H₂O₂. The spectral profile obtained can be understood as a transition from X-Fe⁴⁺ to X-Fe⁵⁺. In order to determine if this oxidation occurred through the intermediate state of X-Fe⁵⁻OH, we performed further spectrophotometric studies in the presence of sodium sulfide (Na₂S). Sodium sulfide adds to a β-δ double bond of a pyrrole and disrupts the porphyrin conjugation, forming a chlorin-type structure (39). Addition of 2 mM Na₂S to a reaction mixture containing intact RBC and glucose + glucose oxidase (as a source of H₂O₂) produced an absorption maximum at 620 nm (Fig. 9B), as expected for the formation of oxsulfito-Hb (39). This spectrum was identical with that obtained upon addition of Na₂S to X-Fe⁴⁻OH (Fig. 9C) and is, therefore, indicative of the presence of X-Fe⁴⁻OH in the RBC treated with a continuous flow of H₂O₂. This technique has previously been used to identify the ferryl state of myoglobin in myocytes that were exposed to H₂O₂ (40). The absence of an observable oxoferryl complex, in the absence of Na₂S, as an intermediate in the oxidation of Hb by H₂O₂ (Fig. 9A) can be explained in terms of the presence of (a) a high concentration of X-Fe⁴⁺ which would favor Reaction 5, and (b) the presence of compounds that might act as reductants for the ferryl complex (i.e. ascorbic acid). The rates of X-Fe⁴⁻OH formation in intact human, bovine, and rabbit RBC were found to be similar (Fig. 10). It is important to note that the primary sequence of the α-chains of human, bovine, and rabbit Hb in the region of Tyr-42 is highly conserved. Thus, the comproportionation reaction between X-Fe⁴⁺ and X-Fe⁵⁻OH appears to be of general significance in RBC.

Significance of the Comproportionation Reaction—In order to determine if the production of X-Fe⁵⁺ in RBC is mainly due to the reaction of X-Fe⁴⁻OH with intracellular reductants (rather than the reaction of X-Fe⁴⁻OH with X-Fe⁴⁺), we measured the rate constants between various reduced intracellular compounds (NADH, NADPH, glutathione, ascorbic acid, and uric acid) and both bovine and human X-Fe⁴⁻OH (Table I). Respectable rate constants for reaction of X-Fe⁴⁻OH with Hb, uric acid, and ascorbic acid were observed, although no reduction of X-Fe⁴⁻OH by glutathione, NADH, or NADPH could be detected (Table I). When the intracellular RBC concentrations of urate, ascorbate, and Hb are taken into account, it is clear that X-Fe⁴⁺ represents, by far, the most significant potential reductant for X-Fe⁵⁻OH (Table I). These results further strengthen our proposal that the reaction between X-Fe⁴⁺ and X-Fe⁵⁻OH may represent a significant antioxidant capacity in the RBC. Importantly, Table I also demonstrates that the reaction of Hb with X-Fe⁴⁺ to generate X-Fe⁵⁻OH occurs with a much higher yield than pure H₂O₂. Thus, the results of Fig. 1 cannot be ascribed to any complex interactions between Hb and other components or products (e.g. gluconic acid) of the glucose + glucose oxidase system.
A Novel Antioxidant Role for Hemoglobin

FIG. 9. Spectrophotometric determination of $X\text{-Fe}^{IV}$-OH and $X\text{-Fe}^{III}$ generation in intact human RBC exposed to a continuous flux of $H_2O_2$. Panel A, RBC (0.3%) in Krebs-Ringer phosphate buffer (pH 7.4) plus 10 mM glucose and 7.5 $\mu$g/ml glucose oxidase were incubated at 25 $^\circ$C. Panel B, experimental conditions as in panel A, except for the addition of Na$_2$S (2 mM). In panels A and B, repetitive scans were recorded each minute over a 30-min incubation. Panel C, $X\text{-Fe}^{IV}$-OH was obtained by adding $H_2O_2$ to $X\text{-Fe}^{III}$ in a molar ratio of 5:1. Catalase (2 $\mu$m) was added to decompose excess $H_2O_2$, and Na$_2$S (2 mM) was added to obtain the sulfoderivative.

Fig. 10. Rates of $X\text{-Fe}^{IV}$-OH formation in intact human, bovine, and rabbit RBC exposed to a continuous flux of $H_2O_2$. Human, bovine, and rabbit RBC were exposed to $H_2O_2$ as described in the legend to Fig. 9B. The formation of $X\text{-Fe}^{IV}$-OH was assessed, following addition of Na$_2$S, as described in the legend to Fig. 9B. Formation of the sulfoderivatives is reported at 630 nm absorbance.

peroxidase with $H_2O_2$ (3.4 $\times$ 10$^5$ (41) and 0.6–1.8 $\times$ 10$^8$ M$^{-1}$ s$^{-1}$ (42), respectively) would indicate that these enzymes should contribute more to the removal of $H_2O_2$ from red cells than does Hb (100 M$^{-1}$ s$^{-1}$, Equation 8). Indeed, the rate of $X\text{-Fe}^{III}$ production in the experiments of Fig. 8 indicates that Hb accounts for some 4% of the total $H_2O_2$ catabolism of red cells. Thus, although Hb does not appear to be a major competitor for glutathione peroxidase and catalase, our results do indicate a highly effective antioxidant activity for $X\text{-Fe}^{IV}$O$_2^*$ resulting in the harmless reduction of $X\text{-Fe}^{IV}$-OH to $X\text{-Fe}^{III}$ (Scheme II). In this scheme, $X\text{-Fe}^{III}$ is continuously re-reduced to $X\text{-Fe}^{IV}$O$_2^*$ by methemoglobin reductase (Scheme II).

Our results also have significance for myoglobin, which is present in high concentrations in cardiac muscle (about 1 mM in myocytes) and could form ferrylmyoglobin during oxidative stress, e.g. in ischemia-reperfusion injury (5). Previously, Whitburn (44) reported a similar comproportionation reaction between oxymyoglobin and ferrylmyoglobin. Although the mechanism was not determined in Whitburn's work, electron transfer mediated by an amino acid in the vicinity of the heme moiety was suggested (44). We have successfully repeated Whitburn's experiments and found that a comproportionation reaction between oxymyoglobin and ferrylmyoglobin does, indeed, occur. We have also performed limited tyrosine acetylation studies with myoglobin and have found

### TABLE I

<table>
<thead>
<tr>
<th>Addition</th>
<th>$k$ (M$^{-1}$ s$^{-1}$)</th>
<th>Concentration in RBC (dM/dt)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GSH</td>
<td>ND</td>
<td>0</td>
</tr>
<tr>
<td>NADH</td>
<td>ND</td>
<td>0</td>
</tr>
<tr>
<td>NADPH</td>
<td>ND</td>
<td>0</td>
</tr>
<tr>
<td>Uric acid</td>
<td>153</td>
<td>1.3 $\times$ 10$^{-4}$</td>
</tr>
<tr>
<td>Ascorbic acid</td>
<td>15</td>
<td>3.5 $\times$ 10$^{-5}$</td>
</tr>
<tr>
<td>Bovine $X\text{-Fe}^{III}$O$_2^*$</td>
<td>19</td>
<td>5.0 $\times$ 10$^{-5}$</td>
</tr>
<tr>
<td>Human $X\text{-Fe}^{III}$O$_2^*$</td>
<td>23</td>
<td>5.0 $\times$ 10$^{-3}$</td>
</tr>
</tbody>
</table>

* Intracellular concentrations correspond to Ref. 43.

The $X\text{-Fe}^{IV}$-OH was preformed by adding a bolus of $H_2O_2$ (in a ratio of 5:1) to a solution containing 67 $\mu$M $X\text{-Fe}^{III}$ in Krebs-Ringer phosphate buffer (pH 7.4). The oxidation was stopped by adding catalase (1 $\mu$m) to the medium after 5 min. The transition of $X\text{-Fe}^{IV}$-OH to $X\text{-Fe}^{III}$ was measured at 630 nm, following addition of potential reductants.

The rate constants ($k$) were calculated as the slopes of the linear part of plots of $X\text{-Fe}^{III}$ formation versus concentration of the reductant. ND, not detectable.
that comproportionation is inhibited when tyrosine residues are blocked (data not shown). Thus, tyrosine residues appear to be involved in similar comproportionation reactions with both Hb and myoglobin. Ferrylmyoglobin, in vitro, is regarded as an oxidizing agent capable of promoting the peroxidation of fatty acids and the oxidation of β-carotene, ascorbic acid, and uric acid, as well as other molecules of less biological relevance (11). Although ferrylmyoglobin may, indeed, cause injury to cardiac cells (5) it now appears possible that oxy-myoglobin may minimize such damage by the same comproportionation reaction shown here for Hb.

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