The Reactivity of Thiols and Disulfides with Different Redox States of Myoglobin

REDOX AND ADDITION REACTIONS AND FORMATION OF THIYL RADICAL INTERMEDIATES*

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The reactivity of several thiols, including glutathione, dihydrolipoic acid, cysteine, N-acetyl cysteine, and ergothioneine, as well as several disulfides, toward different redox states of myoglobin, mainly met-myoglobin (HX-FeIII) and ferrylmyoglobin (HX-FeIV=O), was evaluated by optical spectral analysis, product formation, and thiyl free radical generation.

Only dihydrolipoic acid reduced met-myoglobin to oxy-myoglobin, whereas all the other thiols tested did not interact with met-myoglobin. Although the redox transitions involved in the former reduction were expected to yield the dihydrolipoate thiyl radical, the reaction was EPR silent.

Conversely, all thiols interacted to different extent with the high oxidation state of myoglobin, i.e. ferrylmyoglobin, via two processes. First, direct electron transfer to heme iron in ferrylmyoglobin (HX-FeIV=O) with formation of met-myoglobin (HX-FeIII) or oxy-myoglobin (HX-FeII=O); the former transition was effected by all thiols except dihydrolipoate, which facilitated the latter, i.e. the formation of the two-electron reduction product of ferrylmyoglobin. Second, nucleophilic addition onto a pyrrole in ferrylmyoglobin with subsequent formation of sulfmyoglobin. The contribution of either direct electron transfer to the heme iron or nucleophilic addition depended on the physicochemical properties of the thiol involved and on the availability of H2O2 to reoxidize met-myoglobin to ferrylmyoglobin. The thiyl radicals of glutathione, cysteine, and N-acetylcysteine were formed during the interaction of the corresponding thiols with ferrylmyoglobin and detected by EPR in conjunction with the spin trap 5,5'-dimethyl-1-pyrroline-N-oxide. The intensity of the EPR signal was insensitive to superoxide dismutase and it was decreased, but not suppressed, by catalase.

The disulfides of glutathione and cysteine did not react with ferrylmyoglobin, but the disulfide bridge in lipoic acid interacted efficiently with the ferryl species by either reducing directly the heme iron to form met-myoglobin or adding onto a pyrrole ring to form sulfomyoglobin; either process depended on the presence or absence of catalase (to eliminate the excess of H2O2) in the reaction mixture, respectively.

The biological significance of the above results is discussed in terms of the occurrence and distribution of high oxidation states of myoglobin, its specific participation in cellular injury, and its potential interaction with biologically important thiols leading to either recovery of myoglobin or generation of nonfunctional forms of the hemoprotein as sulfmyoglobin.

The oxidation of MbIII by H2O2 yields MbIV, in which the heme iron is one oxidizing equivalent above that of MbIII. A heterolytic mechanism seems to be involved in the formation of MbIV and its EPR-detectable free radical transient form, conventionally formulated as HX-FeIV=O-H or HX-FeIV=O and 'X-FeIV=O-H, respectively (where HX indicates an amino acid residue in the globin) (Reaction 1) (1-6). The radical character in MbIV is centered on aromatic residues, probably on tyrosines (3, 4, 7-10).

\[
\text{HX-Fe}^{III} + \text{H}_2\text{O}_2 \rightarrow \text{H} \rightarrow \text{X-Fe}^{IV}-\text{OH} + \text{H}_2\text{O}
\] (1)

The reduction of MbIV or its transient free radical form, MbV, is a complex process involving steps with both multiple components at different stages of oxidation and reactions which are not necessarily irreversible. This process is expected to be modulated by thermodynamic factors, such as some physicochemical properties of the reductant(s) and the reduction potential of the redox couples involved, as well as by kinetic factors. As a rule, although two-electron reduction products could be isolated, these processes seem to proceed in one-electron transfer steps, involving the transitions in Reaction 2

\[
\text{Mb}^{IV} \text{(or } \text{Mb}^{V}) \rightarrow \text{Mb}^{III} \rightarrow \text{Mb}^{II}=\text{O}_2 \text{(or MbI)}
\] (2)

which, for the particular case of thiols, would be hypothetically coupled to oxidation steps leading to disulfide formation (Reaction 3)

\[
2 \text{RS}^- \rightarrow 2 \text{RS}^+ \rightarrow \text{RSSR}
\] (3)

provided that the experimental conditions permit decay of the intermediate thiyl-free radical by pathways involving its dimerization and/or conjugation with the thiolate anion (RS-) (11). The redox couples in Reactions 2 and 3 reflect only

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1 The abbreviations used are: MbIII or HX-FeIII, met-myoglobin; DMPO, 5,5'-dimethyl-1-pyrroline-N-oxide; MbO2, oxy-myoglobin; MbIV or HX-FeIV-OH ferrylmyoglobin; 'MbV or 'X-FeIV-OH, radical form of ferrylmyoglobin.

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different stages of electron transfer from the thiol to the heme iron in Mb. In addition, thiols may effect electron transfer by a mechanism other than the latter, i.e. sulfur nucleophilic addition to a \( \beta \)-carbon atom of a pyrrole, with both reducing equivalents of the thiol being ultimately transferred to the iron-ligated complex (2, 5, 12, 13). The resulting Mb derivative contains a sulfur bond to the pyrrole in a chlorin-type structure, known as sulfmyoglobin (12, 13).

Among thiol-containing compounds, GSH (14) and ergothioneine (15) reduce Mb\( ^{V} \) to Mb\( ^{III} \), but they cannot accomplish further reduction of the latter to oxy-myoglobin (Mb\( ^{V} \)). Lipoic acid, a disulfide which occurs bound to proteins by forming an amide linkage through its carboxyl group, plays a key catalytic role in biological oxidations where it oscillates between the disulfide, lipoate, and the dithiol, dihydrolipoate. Dihydrolipoate is also known to display an antioxidant activity in connection with vitamin E (16, 17) and the selenocompound ebselen (18), and probably encompassing the redox transition in Reaction 4.

\[
\text{HS-CH}_2\text{CO}- \xrightarrow{\text{S-S}} \text{S}-\text{CH}_2\text{CO}^- + 2H^+ \quad (4)
\]

This study is aimed at a systematic understanding of the molecular mechanism(s) inherent in the interaction of different redox states of Mb with biological thiol-containing compounds with emphasis on dihydrolipoate and its disulfide, lipoate. A comparative study has also been performed regarding the ability of different thiols (a) to effect electron transfer to the high oxidation state of Fe in Mb\( ^{V} \), within a process coupled to the formation of free radical intermediates, i.e. thyl radicals, and (b) to participate in nucleophilic addition onto the heme pyrrole group with consequent formation of sulfmyoglobin.

### MATERIALS AND METHODS

**Chemicals and Biochemicals**—Met-myoglobin (horse heart, type III, 99% purity), DL-6,8-thioctic acid (oxidized and reduced forms), and H\( _2 \)O\( _2 \) were from Sigma. Glutathione, glutathione disulfide, and \( \text{N-acetylimidazole} \) were from Boehringer (Mannheim, Germany). 5,5'-Dimethyl-1-pyrroline-N-oxide (DMPO) was from Aldrich Chemical Co. and was purified by repeated charcoal filtration until the spin trap alone was virtually signal free. A highly purified catalase, used as molecular weight standard for chromatography, was obtained from Sigma (from bovine liver), with essentially the same results. All other chemicals were of reagent grade. The reduced DL-G\( \text{SH} \& \text{thioctic acid solution was kept sealed under argon at 4 °C to prevent its autoxidation.**

**Spectrophotometric Assays**—The standard assay mixture used for spectrophotometric assays in the visible region consisted of 25 \( \mu \)M Mb\( ^{III} \) and 100 \( \mu \)M H\( _2 \)O\( _2 \) in 0.1 M potassium phosphate buffer, pH 7.4, containing 1 mM EDTA, and different concentrations of thiol compounds. The standard assay mixture used for spectrophotometric assays in the Soret region was essentially the same as above, but Mb\( ^{IV} \) concentration was lowered to 10 \( \mu \)M. Assay temperature was 30°C. Myoglobin spectral changes were monitored with either a UV visible double-beam spectrophotometer (Hitachi Instruments, Danbury, CT; model U-3110) or a diode array spectrophotometer (Beckman Instruments, Inc., Fullerton, CA; DU Series 7000) in the 400-700-nm range. Oxy-myoglobin (\( \text{Mb}^{V} \) at 280 nm = 14.4 \( \text{mM}^{-1} \text{cm}^{-1} \)) and myoglobin (\( \text{Mb}^{III} \) at 280 nm = 3.5 \( \text{mM}^{-1} \text{cm}^{-1} \)) concentrations were calculated after the respective extinction coefficients at their wavelengths of maximal absorption in the visible region (19). Ferrylmyoglobin formation was followed at \( \lambda_{435} \text{nm} \), the wavelength at which the absorption spectra of met-myoglobin and ferrylmyoglobin differ most. Total myoglobin, the sum of oxy- and met-myoglobin was calculated according to the following formula: \( \text{[Mb]} = \text{[Mb]}^{V} + \text{[Mb]}^{III} \) = 66 \( \times \text{A}_{280} \) - 50 \( \times \text{A}_{435} \) ([Myoglobin] = 279 \( \times \text{A}_{435} \) - 3.0 \( \times \text{A}_{280} \)).

Dihydrolipoic acid was measured by its stoichiometric reaction with bis-[5-carboxy-4-nitrophenyl]disulfide (molar absorptivity = 13.6 \( \text{mM}^{-1} \text{cm}^{-1} \)).

Addition of dihydrolipoic acid to a solution containing Mb\( ^{III} \) exerted spectral changes which consisted in a rapid decrease in absorbance at 505 and 630 nm and an equally rapid increase at 546 and 580 nm, with isosbestic points at 482, 522, and 601 nm (Fig. 1). The resulting spectral profile was essentially that of Mb\( ^{V} \).O\( _2 \). The reduction of Mb\( ^{III} \) to Mb\( ^{O} \)O\( _2 \) by dihydrolipoate is summarized in Reaction 5 (\( \text{-R} = \text{(-CH}_2\text{-COO)}^- \)). Although the redox couples in Reaction 5, i.e. Mb\( ^{III} \)→Mb\( ^{O} \)O\( _2 \) and dihydrolipoate→lipoate, imply one- and two-electron transitions, respectively, the reaction is EPR silent and no thyl-free radical intermediates could be detected (see below).

\[
2 \text{Mb}^{III} + 2 \text{O}_2 + \text{R} = 2 \text{Mb}^{IV} + \text{R} + 2 \text{H}^+ \quad (5)
\]

Fig. 2A shows the time course of Mb\( ^{III} \) reduction by dihydrolipoate and the ensuing formation of Mb\( ^{O} \)O\( _2 \). Under the experimental conditions in Fig. 2A, product generation pro-
FIG. 2. Time course of oxy-myoglobin formation upon reduction of met-myoglobin by dihydrolipoic acid. A, time course of Mb \textsuperscript{III}O\textsubscript{2} formation (\(A_{580\text{nm}}\)) and Mb \textsuperscript{III} reduction (\(A_{420\text{nm}}\)). Assay conditions as in Fig. 1. B, dependence of the rate of Mb \textsuperscript{III}O\textsubscript{2} formation on dihydrolipoate concentration. Assay conditions as in Fig. 1 with varying concentrations of dihydrolipoate.

TABLE I

<table>
<thead>
<tr>
<th>Rate constants for the reaction of several thiols and disulfides with different redox states of myoglobin</th>
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<tbody>
<tr>
<td>Thiols or disulfide</td>
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<tr>
<td></td>
</tr>
<tr>
<td>Thiols</td>
</tr>
<tr>
<td>Dihydrolipoic acid</td>
</tr>
<tr>
<td>Glutathione*</td>
</tr>
<tr>
<td>Cysteine</td>
</tr>
<tr>
<td>Ergothioneine*</td>
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<table>
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<tr>
<th>Disulfides</th>
<th>(X/2SS)</th>
<th>(2.47 \pm 0.34 \times 10^3)</th>
<th>(1.85 \pm 0.19 \times 10^3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lipoic acid</td>
<td>3.06 (2.09 \times 10^3)</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Glutathione disulfide</td>
<td>ND</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>Cystine</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

\*Although no rates were reported, the reaction of GSH and ergothioneine with Mb\textsuperscript{IV} has been published by Galaris \textit{et al.} (14) and Arduini \textit{et al.} (15). ND, not detectable. \(E^{-}(SH)/2SS-\), reduction potential for the RSH \(\rightarrow\) RSSR reaction. \(E^{-}(SH)/2SS-\) values taken from Jocelyn (53).

ceeded at an initial rate of 903 \(\mu M \times \text{min}^{-1}\). The extent of Mb\textsuperscript{III}O\textsubscript{2} formed upon reduction of Mb\textsuperscript{III} by dihydrolipoate was linearly dependent on increasing concentrations of the di thiol up to 125 \(\mu M\) (Fig. 2B). Beyond the latter concentration no significant increases in the rate of Mb\textsuperscript{III}O\textsubscript{2} formation or total Mb\textsuperscript{III}O\textsubscript{2} formed were observed. The second order rate constant \((k_2)\) for Reaction 5\text{forward} was \((2.47 \pm 0.34) \times 10^4 \text{M}^{-1} \text{ s}^{-1}\) \((n = 4; \text{calculated from linear relationships of rate of Mb}\textsuperscript{III}O\textsubscript{2} \text{formation versus dihydrolipoate concentration}) \(\text{(Table I)}\).

As inferred from the time course in Fig. 2A, the Mb\textsuperscript{III}O\textsubscript{2} formed upon reduction of Mb\textsuperscript{III} by dihydrolipoate is not a stable end molecular product; after \(8 \text{ min}\), a slow and gradual decline in the Mb\textsuperscript{III}O\textsubscript{2} absorption wavelength was observed, which was accompanied by an increase in absorbance at 630 nm. Throughout these changes, the total amount of myoglobin \((i.e. [Mb\textsuperscript{III}O\textsubscript{2}] + [Mb\textsuperscript{III}])\) remained constant \((i.e. \text{Mb} = 25.0 \pm 0.51 \mu M; n = 4)\). This was calculated by means of the expression described under “Materials and Methods”). The formation of Mb\textsuperscript{III} cannot be rationalized in terms of an autoxidation of Mb\textsuperscript{III}O\textsubscript{2} to yield O\textsubscript{2} and, eventually, H\textsubscript{2}O\textsubscript{2} (22, 23), for \(a\) the autoxidation of Mb\textsuperscript{III}O\textsubscript{2} proceeds at a rate far slower than the Mb\textsuperscript{III}O\textsubscript{2} \(\rightarrow\) Mb\textsuperscript{III} changes described here; \(b\) the increase in absorbance at 630 nm was insensitive to superoxide dismutase and catalase, and \(c\) the H\textsubscript{2}O\textsubscript{2} formed during Mb\textsuperscript{III}O\textsubscript{2} autoxidation would be expected to oxidize readily Mb\textsuperscript{III} to a ferryl species (22, 23). The disulfide product, lipoate, however, could oxidize Mb\textsuperscript{III}O\textsubscript{2} as indicated in Reaction 5\text{backward}. This view is substantiated by a rapid Mb\textsuperscript{III} formation upon addition of the disulfide, lipoic acid, to a Mb\textsuperscript{III}O\textsubscript{2} solution (Fig. 3).

In summary, dihydrolipoate reduced rapidly Mb\textsuperscript{III}O\textsubscript{2} to Mb\textsuperscript{III}, and the latter was partly oxidized back to Mb\textsuperscript{III}. The different redox states of myoglobin present in the reaction mixture at equilibrium clearly depend on the accumulation of oxidized product, lipoate. It is worth noting that the reduction of Mb\textsuperscript{III} to Mb\textsuperscript{III}O\textsubscript{2} by dihydrolipoate is a unique feature among thiols, for other compounds, such as cysteine, N-acetylcysteine, GSH, and ergothioneine, cannot accomplish this reaction \(\text{(Table I)}\). It is also worth mentioning that although ergothioneine could not reduce Mb\textsuperscript{III} to Mb\textsuperscript{III}O\textsubscript{2}, its analog compound, 4-mercaptopimidoazolo, was reported to reduce ferricytochrome \(c\) (24).

**Interaction of Dihydrolipoate and Other Thiols with Ferrylmyoglobin**

The addition of a two molar excess of H\textsubscript{2}O\textsubscript{2} to Mb\textsuperscript{III} caused a rapid increase in absorbance in the 520–600 nm region, with main peaks at 548 and 582 nm, and a decrease at 630 nm, with an isosbestic point at 618 nm. This spectral profile is ascribed to Mb\textsuperscript{IV} \(\text{Fig. 4, dotted line)}\) and is indistinguishable from that of its free radical form \(\text{(X-Fe}\textsuperscript{IV}-\text{OH} \text{in Reaction 1)}\).

Because of significant differences in the optical absorption spectra, the interaction of thiols with Mb\textsuperscript{IV} are described below in two conditions, in the presence and absence of catalase.

**Interaction of Thiols with Ferrylmyoglobin in the Presence of Catalase**

Following the addition of catalase to remove the excess of H\textsubscript{2}O\textsubscript{2}, the addition of dihydrolipoic acid to the Mb\textsuperscript{IV} solution resulted in rapid spectral changes with prominent absorptions at 542 and 581 nm, which may be ascribed to Mb\textsuperscript{III}O\textsubscript{2} and a less prominent one or shoulder at around 617 nm \(\text{Fig. 4B)}\). The latter (along with a slight green coloration of the solution) suggested the presence of ferrous sulfmyoglobin. However, this absorption was not sufficiently intense to distinguish the oxy- (623 nm) or deoxy- (616 nm) forms of
ferrous sulfmyoglobin (25). No absorption at 715–717 nm, indicative of the ferric form of sulfmyoglobin (2, 12), was observed. The optical changes in the visible were accompanied by changes in the Soret region: the 421-nm absorption of ferrymyoglobin was shifted initially and transitarily toward 416–417 nm (absorption wavelength of MbO2) (26) and finally toward 411 nm by dihydrolipoate (Fig. 4A). The latter maximum was part of a broad band, probably including a sulfmyoglobin component; alike the changes observed in the visible region, a clear distinction of the contribution of the oxy- (408 nm) and deoxy- (421–424 nm) forms of sulfmyoglobin (2, 12, 25) to the Soret region spectrum was difficult to assess.

\[
\text{Mb}^{IV} \rightarrow \text{Fe}^{V} - \text{OH} + \text{O}_2 + \text{HS} \text{SH} \rightarrow \text{Mb}^{III} \text{O}_2 + \text{H}_2 \text{O} + \text{S-S} \text{R} + \text{H}^+ \tag{6}
\]

![Image](image-url)

**Fig. 3. Oxidation of oxy-myoglobin by lipoic acid.** Assay conditions: 25 μM MbO2 in 0.1 M potassium phosphate buffer, pH 7.4, containing 1 mM EDTA, was supplemented with 0.5 mM lipoic acid. The figure shows repetitive scans initiated 0.5 min after the addition of dihydrolipoic acid. Intervals between successive scans, 1 min. The downward and upward arrows indicate decreasing and increasing absorption as the reaction proceeds.


dihydrolipoate with Mb⁴⁺ yields a composite of MbO₂ and sulfmyoglobin. Although Mb⁴⁺ → Mb⁵⁺O₂ and dihydrolipoate → lipoate are both two-electron redox transitions, it is unlikely that Mb⁴⁺ is reduced directly to MbO₂ via two electrons, for 7 s after the addition of dihydrolipoate to Mb⁴⁺ a composite of Mb⁶⁻ and MbO₂ was observed (not shown). We recognize that Reaction 6 as written need not be correct, but it reflects, regardless of stoichiometric factors, the overall electron-transfer reaction and products detected. Although the transition Mb⁴⁺ → Mb⁶⁻ → MbO₂ is a plausible sequence for electron transfer, thyl-free radical intermediates of dihydrolipoic acid could not be detected (see below).

Alternatively, the possibility that the transient Mb⁶⁻ species could originate from a comproportionation reaction involving electron transfer between Mb⁴⁺ and MbO₂ (27) seems unlikely, for (a) Mb⁴⁺ would be required to be constantly present in the reaction mixture in order to react with MbO₂, and (b) o-acetylation of the tyrosyl residues in Mb (21), presumably involved in the electron transfer in the comproportionation reaction (27), did not prevent the transient formation of Mb⁶⁻ (not shown).

Reaction 6 does not regard though the formation of sulfmyoglobin, which is responsible for the absorption at around 617 nm and the broad Soret band with a maximum at 411 nm. The exact chemical formulation of sulfmyoglobin is not entirely considered in Reaction 7 in which the terms Mb-S-Fe₆⁰ and Mb₅-Se³⁺O₂ are only abbreviations. Considerable information has been obtained about the electronic structure of sulfmyoglobin: the molecular mechanism for its formation requires the reaction of the ferryl compound with inorganic sulfide, involving a nucleophilic attack of the thiol on a β-carbon atom of a pyrrole, with both reducing equivalents of the thiol being ultimately transferred to the iron-ligand complex (2, 5, 12, 13, 25). It is not conceivable that the observed MbO₂ originates from a decomposition of sulfmyoglobin by H₂O₂ (2), for the excess of the latter is reduced by catalase under these experimental conditions.

\[
\text{Mb}^{IV} - \text{Fe}^{V} - \text{OH} + \text{SH} \rightarrow \frac{\text{Mb-S-Fe}^{VI}}{\lambda_{618}, \lambda_{421-424}} + \text{H}_2 \text{O} \tag{7}
\]

It is worth noting that the other thiol compounds tested, i.e. GSH, cysteine, N-acetylcyisteine, and ergothioneine, cannot reduce Mb⁴⁺ to MbO₂, but to Mb⁶⁻ (Table I). The interaction of GSH (14) and ergothioneine (15) with Mb⁴⁺ has been previously described, although no rates were reported. Cysteine efficiently reduces Mb⁴⁺ to Mb⁶⁻ ((2.19 ± 0.28) × 10⁷, Table I) as indicated by the optical absorption spectra in Fig. 6. At variance with dihydrolipoic acid, the cysteine-mediqated reduction of Mb⁴⁺ is not associated with a clear increase in absorbance at 616 nm (indicative of ferrous sulfmyoglobin), but at 626 nm (Fig. 6B). The absorption changes in the Soret region showed that cysteine, upon its reaction with Mb⁴⁺, exerted a transitory shift from 421 nm (absorption wavelength of Mb⁴⁺) toward 409 nm (absorption wavelength of Mb⁶⁻) along with a pronounced shoulder at 421 nm (Fig. 6A, trace a); with time, the latter shoulder decreased and absorption at 409 nm increased (Fig. 6A, trace b). The final
FIG. 4. Reduction of ferrylmyoglobin by dihydrolipoic acid. A, Soret region spectral changes. Assay conditions: 10 \mu M \text{Mb}^{IV} (- - -) in 0.1 M potassium phosphate buffer, pH 7.4, containing 1 mM EDTA, was supplemented with 20 \mu M \text{H}_{2}\text{O}_{2} to generate ferrylmyoglobin (\text{Mb}^{V}) (---). Following the addition of 400 units of catalase \times ml\(^{-1}\) to remove excess of \text{H}_{2}\text{O}_{2}, 100 \mu M dihydrolipoate was added to the reaction mixture, resulting in a progressive spectral shift from 421 toward 411 nm (---, traces a-c). B, visible region spectral changes. Assay conditions: 25 \mu M \text{Mb}^{IV} (- - -) in 0.1 M potassium phosphate buffer, pH 7.4, containing 1 mM EDTA, was supplemented with 50 \mu M \text{H}_{2}\text{O}_{2} to generate ferrylmyoglobin (\text{Mb}^{V}) (---). Following the addition of 400 units of catalase \times ml\(^{-1}\) to remove excess of \text{H}_{2}\text{O}_{2}, 9.0 \mu M dihydrolipoate was added to the reaction mixture, resulting in the immediate formation of \text{Mb}^{V}_{2}O_{2} (---).

FIG. 5. Time course of \text{Mb}^{V}_{2}O_{2} formation and dihydrolipoate consumption during the reaction of dihydrolipoate with \text{Mb}^{V}. Assay conditions, 0.25 mM dihydrolipoate was added to a solution containing ferryl-myoglobin (\text{Mb}^{V}), generated upon the reaction of 50 \mu M \text{Mb}^{IN} plus 0.1 mM \text{H}_{2}\text{O}_{2} and followed by addition of 400 units of catalase \times ml\(^{-1}\)). Dihydrolipoate concentration was assayed as described under "Materials and Methods."

Interaction of Thiols with Ferrylmyoglobin in the Absence of Catalase—The addition of 5–10 molar excess of \text{H}_{2}\text{O}_{2} to \text{Mb}^{IV} produced \text{Mb}^{III} (Fig. 7) with the absorption spectral characteristics previously described in Fig. 4 (dotted line). In the absence of catalase, dihydrolipoate exerted different spectral changes from those reported above in the presence of the enzyme (Fig. 4). The new spectrum in the visible region was initially characterized by maximal absorption at 542 and 580 nm and a shoulder at around 620 nm (Fig. 7B). With time the intensity of these absorption wavelengths decreased, resulting in a loss of structure of the spectrum. No absorption was detected at 675 nm, which could be ascribed to a ferric sulfymyoglobin hydroxide (28). The Soret region spectrum did not show any broadening and was identical to that of the native \text{Mb}^{III}.

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FIG. 6. Soret and visible absorption spectral changes during the reaction of cysteine with ferrylmyoglobin in the presence of catalase. A, Soret region spectral changes. Assay conditions: 10 \mu M \text{Mb}^{IV} (-- -- --, \text{A}_{max} = 409 nm) in 0.1 M potassium phosphate buffer, pH 7.4, containing 1 mM EDTA, was supplemented with 100 \mu M \text{H}_{2}\text{O}_{2} to form \text{Mb}^{V} (-- -- --, \text{A}_{max} = 421 nm). Following the addition of catalase (400 units \times ml\(^{-1}\)) to destroy the excess of peroxide, 400 \mu M cysteine was added (solid lines). a and b correspond to absorption spectra taken 1 and 10 min after the addition of cysteine. B, visible region spectral changes. Assay conditions: as in A but concentrations of \text{Mb}^{IV} and \text{H}_{2}\text{O}_{2} were 25 \mu M and 0.25 mM, respectively. 2 mM cysteine was added to \text{Mb}^{IV} and repetitive scans taken every minute. The downward and upward arrows indicate decrease or increase in absorbance.

FIG. 7. Optical spectral changes of ferrylmyoglobin following its interaction with dihydrolipoic acid in the absence of catalase. Assay conditions as in Fig. 4, but in the absence of catalase. Repetitive scans were initiated 0.5 min after the addition of dihydrolipoic acid. Intervals between successive scans, 1 min. The downward arrows indicate decreasing absorption as the reaction proceeds.
502 and 580 nm with shoulders at around 540 and 620 nm (Fig. 8B). The absorption changes in the Soret region did not reveal a clear spectral profile which could be ascribed to MbH3, but a progressive shift of the 421-nm absorption toward shorter wavelengths (411 nm) along with a decrease in intensity (Fig. 8A, solid lines a-c).

**Reaction of the Disulfide Lipoate with Ferrylmyoglobin**

The disulfide lipoate reduced MbIV to MbH3 as shown by the visible absorption spectral changes in Fig. 9. The final spectrum showed peaks at 502 and 580 nm and a shoulder at 540 nm. The expected absorbance increase at 630 nm, characteristic of MbH3, however, was not observed; instead, increasing absorption at 620 nm, characteristic of sulfmyoglobin, was detected.

The reaction of lipoic acid with ferrylmyoglobin proceeded with a second order rate constant of $(3.06 \pm 0.69) \times 10^3$ M$^{-1}$ s$^{-1}$ (Table I). The mechanism supporting the reduction of MbIV by the disulfide is not clear and hypothetically it may involve oxidation of the S-S bond to sulfonic acid.

Spectral changes in the Soret region recorded in the presence of catalase were similar to those described for cysteine (Fig. 6), albeit less rapid: a progressive shift from 421 toward 409 nm, along with a broadening of the latter absorption band (respective to that of MbH3). In the absence of catalase, lipoate failed to promote the $421 \rightarrow 409$-nm shift but caused a decrease in intensity and broadening of the 421-nm band. These results were similar to those described in Fig. 7 for the reaction of dihydrolipoate with MbIV in the absence of catalase.

At variance with dihydrolipoate, the corresponding disulfide could not reduce MbH3 to MbO2. It is worth noting, that other disulfides, such as GSSG and cystine, do not interact with MbIV, as inferred by the lack of changes in the absorption spectrum of the latter upon incubation with those disulfides.

**Formation of Thiyl-free Radical Intermediates during the Reaction of Thiols with Ferrylmyoglobin**

Thiol oxidation is a feature accompanying several free radical and/or enzymic reactions. In some instances, the formation of the corresponding disulfide has been shown to proceed with intermediate formation of thyl radicals (RS$^\cdot$), which can be readily detected by using EPR spectroscopy in conjunction with the spin trap DMPO (29).

Among the thiol compounds tested (listed in Table I), except dihydrolipoic acid, the other aliphatic thiols, i.e. GSH, cysteine, and N-acetylcysteine, produced thyl radicals upon their individual reactions with MbIV (Fig. 10). Incubation of MbIV with GSH and the spin trap DMPO yielded the characteristic four-line spectrum of the DMPO/GSH thyl radical adduct (hyperfine splitting constants: $a_N = 15.4$ G, $a_H = 16.2$ G) (30) (Fig. 10A). Experiments under similar conditions with cysteine (Fig. 10B) (hyperfine splitting constants: $a_N = 15.3$ G, $a_H = 17.0$ G) (31) or N-acetylcysteine (Fig. 10C) (hyperfine splitting constants: $a_N = 15.0$ G, $a_H = 16.8$ G) (32) yielded the corresponding DMPO thyl radical adduct. At variance with what was reported on thyl radical formation during the lactoperoxidase reaction (33), in our experimental conditions N-acetylation of cysteine did not impair its oxidation to the corresponding thyl radical. In the three instances above, the DMPO/thyl radical was the sole free radical adduct detected. No DMPO adducts of $O_2^-$ or HO$^-$ were observed. The EPR signals were insensitive to superoxide dismutase and their intensity was decreased, but not suppressed, by catalase (see below).

The formation of thyl radicals arising from the reaction of MbIV with the above-mentioned aliphatic thiols is consistent with a mechanism involving electron transfer from the thiol to the high oxidation state of Fe in Mb (Reaction 8). In addition to the latter, another center for thiol reactivity and electron transfer may occur in the Mb molecule, i.e. the amino acid-centered free radical (X$^\cdot$ in Reaction 9); this reaction...
seems unlikely in experimental models containing catalase or with [H2O2]/[MbIII] ratios < 1.

\[
\text{HX-Fe}^\text{V}-\text{OH} + \text{RS}^- (+\text{H}^+) \rightarrow \text{HX-Fe}^\text{III} + \text{H}_2\text{O} + \text{RS}^- \quad (8)
\]

\[
\text{'X-Fe}^\text{V}-\text{OH} + \text{RS}^- (+\text{H}^+) \rightarrow \text{HX-Fe}^\text{V}-\text{OH} + \text{RS}^- \quad (9)
\]

On the other hand, the reactions of dihydrolipoic acid and ergothioneine with MbIV were EPR silent. The former dithiol failed to produce any EPR signal upon its reaction with MbIII and MbIV in the presence of DMPO, despite the fact that the first redox transition is a one-electron transfer process. This could be explained by the close proximity of the SH groups in dihydrolipoic acid, which upon oxidation to S- rapidly dimerize to the disulfide, making their trapping by DMPO virtually impossible. Conversely, the absence of an EPR signal arising from the reaction of ergothioneine with MbIII was expected, for aromatic thiols, which are highly resonance-stabilized, do not react with DMPO.

As stated above, catalase did not abolish the signal corresponding to the DMPO/RS'- adduct, but it only decreased its intensity. The higher intensity signal in the absence of the enzyme is an expected feature, which can be accounted for in terms of a redox cycle involving Reactions 1 and 8 above, along with a potential reactivity of the thiol toward the protein radicals probably centered at a tyrosine residue (Reaction 9) (7-10).

In every instance, regardless of the presence or absence of catalase, the intensity of the DMPO/RS'- adduct of the mentioned aliphatic thiols increased with increasing thiol concentrations (this is illustrated for the case of cysteine in the inset in Fig. 11). Conversely, the first order rate constant for the decay of the DMPO/cysteiny1 radical adduct decreased with increasing concentrations of the thiol.

**CONCLUDING REMARKS**

**Reaction Mechanisms**—The reactions of thiols with different redox states of Mb are complex as indicated by (a) the variety of myoglobin derivatives originating from this reaction and (b) the difficulty in establishing a general mechanism for thiol reactivity. These observations support the view that the thiol reactivity described here is a function of the physico-chemical properties of the thiol and the redox state of myoglobin and that both these parameters will determine the prevalent reaction mechanism(s).

At least two different reaction mechanisms can be invoked in order to account for the myoglobin derivatives formed: on one hand, sole electron transfer from the thiol to the heme iron (regardless whether these transitions involve one- and two-electron transfer steps) and, on the other hand, transfer of reducing equivalents from the thiol to the iron-ligand complex subsequent to a sulfur nucleophilic attack on a pyrrole. Depending on the chemistry of the sulfur compound, the former process facilitates the reduction of MbIV to MbIII or MbIII to MbO2. These possibilities are summarized in Table I along with the rate constants of these reactions in the presence of catalase and in Reactions 10a and b below for the case of redox transitions involving ferrylmyoglobin (HX-FeV-OH) and resulting in thyl radical (-S') formation. The latter process, although also reductive in nature, implies the formation of new derivatives (i.e. a sulfur bound to a beta-carbon of a pyrrole (12)) other than sole iron valence changes and is expressed in some instances as sulfmyoglobin formation (Reactions 10c) with a characteristic absorption at about 620 and 421 nm in the visible and Soret regions, respectively. The abbreviations used in Reaction 10 do not distinguish between the oxy- and deoxy- forms of ferrous myoglobin or ferrous sulfmyoglobin.

\[
\text{HX-Fe}^\text{IV}-\text{OH} + \text{SH} \rightarrow \begin{cases} 
\text{HX-Fe}^\text{III} + \text{S}^- + \text{H}_2 \\
\text{HX-Fe}^\text{III} + \text{S}^- + \text{H}_2 \\
\text{HX-S}^- + \text{Fe}^\text{III} + \text{H}_2\text{O} 
\end{cases} \quad (10a, 10b, 10c)
\]

Of note, the optical spectral analysis shown in this study corresponds to composites of two or more species of the hemoprotein since no purification of the individual myoglobins was carried out. The above reaction mechanisms contribute to different extent to the thiol/Mb redox interactions under different experimental conditions.

1) Dihydrolipoate is the only thiol which can facilitate the MbIII → MbO2 transition. This reaction implies apparently sole electron transfer from the thiol to the heme iron without participation of sulfur nucleophilic addition, in agreement with the requirement of MbIV for sulfmyoglobin formation (2, 5).

2) Lipoate is the only disulfide which can reduce MbIV to
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FIG. 11. Effect of catalase on DMPO-cysteine thiol radical adduct formation produced during the reduction of ferrylmyoglobin. Assay conditions: A, 50 μM met-myoglobin and 80 mM DMPO in 0.1 M potassium phosphate buffer, pH 7.4, containing 1 mM EDTA, were supplemented with 0.5 mM H₂O₂ to initiate the reaction. After 1 min, 2 mM cysteine was added to the formed ferrylmyoglobin and the EPR spectrum was recorded. B, as in A but in the presence of catalase (400 units/ml). Inset: dependence of first order rate constant of decay of an EPR intensity signal of DMPO-cysteine thiol radical adduct on cysteine concentration. Assay conditions as in B. Instrument conditions: receiver gain = 1 × 10⁷; microwave power = 20 milliwatts; microwave frequency = 9.81 GHz; modulation amplitude: 0.963 G; time constant = 1.3 s; scan time = 5.6 min.

The high reactivity toward electrophiles of the disulfide bond of the dithiolane ring in lipoic acid over that of the disulfide bond in open chain disulfides, such as GSSG and cystine, seems to be a function of the lower activation energy caused by the ring strain in the former (34).

3) In the presence of catalase, to remove excess of H₂O₂ and prevent the redox cycle encompassing Reactions 1 and 8, two different reaction mechanisms participate in the thiol/Mb redox transitions and would account for the variability in the yield of the myoglobin species.

(a) Dihydrolipoate, which is the only thiol which can reduce Mb⁴⁺ to Mb³⁺O₂, also favored the formation of sulfmyoglobin; hence, electron transfer and sulfur nucleophilic addition appear to be operative.

(b) The other thiols tested, i.e., GSH, cysteine, N-acetylcysteine, and ergothioneine, can reduce Mb⁴⁺ to Mb³⁺O₂ without formation of sulfmyoglobin as indicated by the visible and Soret region spectral analysis. Hence, the reaction of these thiols with Mb³⁺O₂ seems to involve only electron transfer to the high oxidation state heme iron. The oxidation of GSH, cysteine, and N-acetylcysteine coupled to the reduction of Mb⁴⁺ proceeds with formation of thyl radicals. The reactions leading to thyl radical production are expected to proceed by sole electron transfer from the thiol to the high oxidation state of iron (e.g., Reaction 10b).

4) In the absence of catalase, the redox cycle encompassing Reactions 1, 8, and 9 is operative and, depending on the [H₂O₂]/[Mb³⁺] ratio, the free radical form of Mb⁴⁺ (X–Fe⁴⁺-OH) represents an additional center for electron transfer and thyl reactivity (Reaction 9).

(a) Under these conditions and with all thiols tested, sulfur nucleophilic addition and, hence, the formation of sulfmyoglobin, seems to occur as inferred by the 620-nm peak and the broadening of the Soret region band comprising the 421-nm absorption. The visible absorption spectrum revealed that dihydrolipoate exerted profound changes involving major alterations in the Mb structure.

(b) Thyl-free radical formation is substantially higher in the absence of catalase and the origins of the DMPO/thyl radical adduct probably involve reactions of the thiol with the high oxidation state heme iron and the protein radical centered at a tyrosine residue within the cycle described above.

It is worth noting that the detection of thyl radicals further substantiates the occurrence of a mechanism other than sulfur nucleophilic addition to account for the spectral changes described. Thyl radical formation is satisfactorily explained on thermodynamic and kinetic grounds upon reaction of the thiol with either Mb⁴⁺ or its free radical form (Reactions 8 and 9).

Biological Significance: The biological significance of these studies requires evaluation of (a) the occurrence of ferrylmyoglobin in vivo, (b) its possible pathophysiological role and, hence, the oxidative reactions it might trigger, and (c) the mechanism(s) participating in its recovery encompassing the Mb⁴⁺ → Mb³⁺ in vivo or Mb³⁺ in vivo → Mb⁴⁺ transitions.

Ferrylmyoglobin has been found in vivo (35, 36) and visualized by reflectance spectroscopy in rat diaphragm (37) and the isolated ischemic rat heart (38) after its derivatization with Na₂S to form sulfmyoglobin. Mb⁴⁺ is readily reduced to Mb³⁺ by ascorbate (39); this, along with the demonstration of a ferryl species of Mb in vivo (37, 38) and the reported protective role for ascorbate in induced ischemic arrest associated with cardiopulmonary bypass (40), suggests a new model for ischemia reperfusion, whereby Mb⁴⁺ would play a key role (41).

The chemical reactivity of the ferryl species has been substantiated by a variety of studies involving peroxidation of fatty acids (42–44), oxidation of β-carotene, cholesterol, quinones and quinone-thioether derivatives, and uric acid (45–49), as well as epoxidation of styrene (50). It could be speculated that ferryl complexes, due to their ubiquitous distribution in mammalian tissues, may play a role in redox processes leading to cell injury.

Conversely, it has been proposed that cellular Mb, in the presence of nonenzymic reducing agents, may protect cells against the deleterious effects of oxidizing radicals and H₂O₂ (51). This concept was strengthened by the recovery of Mb⁴⁺ to Mb³⁺ within a process of cycling characteristics (Mb⁴⁺ ↔ Mb³⁺), which involved the reduction of H₂O₂ and the oxidation of biological reducing agents, such as the thiols described here. Such a cycle would restore the O₂-binding capacity of the heme protein and prevent its peroxidatic activity and subsequent cell damage. This cycle is essentially different from the recently described antioxidant role of oxyhemoglobin in red blood cells, which is dependent on a comproportionation reaction of this species with ferrylhemoglobin to yield the met derivative (52).

It is clear from previous studies (2, 5, 12, 13) and this one...
that the high oxidation state of Mb, i.e., ferrymyoglobin or MbIV, is a requisite condition for the formation of sulfmyoglobin upon reaction of the former with thiols. This study also shows that the interaction of thiols with MbIV is a double-edged sword, inasmuch as this reaction can facilitate either the reduction of MbIV to MbIII or the formation of a new derivative, sulfmyoglobin. The prevalence of either reaction seems to depend in particular on the physicochemical properties of the sulfur compound and, in general, on the steady-state concentration of H$_2$O$_2$. Provided that suitable reversion processes are not operative (13), sulfmyoglobin could be considered a nonfunctional hemoprotein, for, although O$_2$ binds reversibly to ferrous sulfmyoglobin, its affinity is 2.5 × 10$^{-6}$-fold decreased relative to myoglobin due to the lowering of electron density at the iron of the prosthetic group (25).

REFERENCES