Pulse Radiolysis Studies of the Reactions of Carbonate Radical Anion with Myoglobin and Hemoglobin

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The reactions of carbonate radical anion [systematic name: trioxidocarbonate(•1−)] with different forms of myoglobin and hemoglobin were studied by pulse radiolysis in N_2O-saturated 0.25 M sodium bicarbonate solutions at pH 10.0 and room temperature. The reactions of $CO_3^{\bullet-}$ with metMb and metHb involve only amino acid residues of the globin and no oxidation of the iron is observed. The second-order rate constants measured are $(4.7 \pm 0.3) \times 10^7$ and $(1.9 \pm 0.3) \times 10^8$ M^{-1} s^{-1}, for metMb and metHb, respectively. The carbonate radical anion-mediated oxidation of oxyHb proceeds in two steps: First, $CO_3^{\bullet-}$ generates radical(s) in the globin which then, over a longer time scale, oxidize the iron center to finally produce ∼40% of metHb. The rate constants obtained for the two steps are $(2.1 \pm 0.1) \times 10^8$ and $(1.0 \pm 0.2) \times 10^2$ s^{-1}, respectively. For the reaction between $CO_3^{\bullet-}$ and oxyMb, at all wavelengths studied we obtained kinetic traces that could be fitted to a single-exponential expression. Two distinct two step mechanisms were proposed to explain the kinetic data. The reaction of $CO_3^{\bullet-}$ with oxyMb proceeds either according to a mechanism identical to that observed for the reaction with oxyHb but with a significantly faster rate of electron transfer from the globin radical(s) to the iron ($>6 \times 10^4$ s^{-1}) or according to a concurring mechanism in which $CO_3^{\bullet-}$ oxidizes directly both ∼50% of the iron center and amino acid residue(s) of the globin.

Introduction

Carbonate radical anion [$CO_3^{\bullet-}$, systematic name: trioxidocarbonate(•1−)] has recently been proposed to be generated in biological systems and has thus started to attract the interest of biochemists.[1] This radical is formed by one-electron oxidation of carbonate (CO_3^{2-}) or bicarbonate (HCO_3^-), but only few biologically relevant compounds are oxidants strong enough to carry out this reaction. Indeed, the reduction potential of $CO_3^{\bullet-}$ is 1.59 V at pH 12.5[2] and 1.78 V at pH 7.0.[3] Besides from the reaction of CO_3^{2-}/HCO_3^- with the hydroxyl radical (see below, reactions 3 and 4),[4] $CO_3^{\bullet-}$ is generated from homolytic O−O bond cleavage of $ONOOCO_2^-$, the adduct produced from the reaction of CO_2 with $ONOO^-$.[5,6] $CO_3^{\bullet-}$ has unambiguously been detected by EPR spectroscopy from decomposition of this adduct,[5,6] but its quantification is still a matter of discussion. It has been proposed that 30%[7,8] or less than 5%[9] of $ONOOCO_2^-$ decays to form the radicals $NO_2^{\bullet}$ and $CO_3^{\bullet-}$. Moreover, it has recently been established that the reduced Cu(I) form of Cu,Zn-superoxide dismutase reacts with hydrogen peroxide to produce a strong oxidant capable of oxidizing CO_3^{2-}/HCO_3^- to $CO_3^{\bullet-}$, which can inactivate the enzyme by oxidation of an adjacent histidine residue or diffuse out of the active site of the protein and oxidize a variety of exogenous substrates.[10−12]

We have investigated the reactions of peroxynitrite with the iron(III) and the oxygenated forms of myoglobin (Mb)[13] and hemoglobin (Hb), in the presence and absence of carbon dioxide.[14−17] The mechanisms of these reactions are complex[18,19] and, for the systems in the presence of CO_2, it is likely that they partly involve reactions of the proteins with $CO_3^{\bullet-}$.[16,17,20,21] In fact, Minetti and co-workers detected by EPR spectroscopy a signal assigned to a tyrosyl centered radical(s) very likely to be produced from the reaction of Hb with $CO_3^{\bullet-}$ upon mixing both oxyHb and metHb with peroxynitrite in the presence of 1.2 mM CO_2.[20,21]

In this paper, we have examined by pulse radiolysis the reactivity of different forms of Mb and Hb toward the carbonate radical anion. Our data show that the reaction of $CO_3^{\bullet-}$ with the iron(III) forms of the proteins involves only the amino acids of the globin, whereas oxyMb and oxyHb are oxidized by $CO_3^{\bullet-}$ to 40−50% of their corresponding iron(III) forms.

Experimental Section

Chemicals. All chemicals used were of the highest purity available. Purified human oxyHb stock solution HbA_0 (57 mg/mL solution with approximately 1.1% metHb) was a kind gift from APEX Bioscience, Inc. Horse heart myoglobin was purchased from Sigma. Concentrated oxyMb, metHb, and metMb solutions were prepared in 0.1 M potassium phosphate buffer pH 7.0 as described previously.[15] Sodium bicarbonate was purchased from Merck.

Methods. Pulse radiolysis experiments were carried out by irradiation of the samples with a 2 MeV electron accelerator (Febetron 705, Hewlett-Packard) as described earlier.[22] The light source was a Xenon-lamp and the optical path length was 1 cm. The detection system consisted of a SpectraPro-300i monochromator and a Hamamatsu R928 photomultiplier. The dose per pulse used varied from 5 to 15 Gy. Dosimetry was conducted by using the thiocyanate dosimeter. All reactions were investigated at different wavelengths in the approximate range 400−600 nm. The kinetics of the reaction of $CO_3^{\bullet-}$ with metMb, metHb, and oxyHb were studied at 600 nm, whereas the rate constant of the reaction with oxyMb was determined by following the absorbance changes at 544 nm.

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Generation of the Carbonate Radical Anion. $CO_3^{\bullet-}$ was generated by irradiating a N_2O-saturated (24.4 mM) 0.25 M sodium bicarbonate solution containing different amounts of protein (pH 10.0). The dose was set to produce between 3 and 10 μM of $CO_3^{\bullet-}$, depending on the protein concentration. Under these conditions the following reactions take place:

$$H_2O \rightarrow H^{\bullet}\ (0.6),\ HO^{\bullet}\ (2.7),\ e_{aq}^{-}\ (2.7),\ H_3O^{+}\ (2.7),\ H_2O_2\ (0.7),\ H_2\ (0.45) \quad (1)$$

$$N_2O + e_{aq}^{-} \rightarrow N_2 + HO^{-} + HO^{\bullet} \quad (2)$$

$$HCO_3^{-} + HO^{\bullet} \rightarrow CO_3^{\bullet-} + H_2O \quad (3)$$

$$CO_3^{2-} + HO^{\bullet} \rightarrow CO_3^{\bullet-} + HO^{-} \quad (4)$$

$$CO_3^{2-} + H^{\bullet} \rightarrow HCO_3^{-} + e_{aq}^{-} \quad (5)$$

$$N_2O + H^{\bullet} \rightarrow N_2 + HO^{\bullet} \quad (6)$$

The second-order rate constants for reactions 2−4 are $k_2 = 9.1 \times 10^9$ M^{-1} s^{-1},[23] $k_3 = 8.5 \times 10^6$ M^{-1} s^{-1},[4] and $k_4 = 4.2 \times 10^8$ M^{-1} s^{-1}.[4] OxyMb and metMb have been reported to react with $HO^{\bullet}$ at rates of $\sim 1 \times 10^9$ and 8×10^9 M^{-1} s^{-1}, respectively.[24,25] At pH 10.0 the concentrations of HCO_3^{-} and CO_3^{2-} in the 0.25 M bicarbonate solution are 0.17 and 0.08 M, respectively (calculated by using the value of 10.33 for the pK_a of HCO_3^{-}).[26] Thus, the experimental conditions chosen ensured that practically all $HO^{\bullet}$ produced from radiolysis of the aqueous solution reacted directly with CO_3^{2-}. Indeed, the observed rate constants for the reactions of $HO^{\bullet}$ with HCO_3^{-} and CO_3^{2-} are 1.4×10^6 and 3.4×10^7 s^{-1}, respectively, whereas those for the reactions of $HO^{\bullet}$ with the highest oxyMb and metMb concentrations used ($\sim$100 μM) are $\sim 10^5$ and 8×10^5 s^{-1}, respectively. $H^{\bullet}$ may react with HCO_3^{-} (4.4×10^4 M^{-1} s^{-1} at pH 8.0 and 273 K) probably forming an intermediate adduct,[27] with HO^{-} (to generate hydrated electrons with a rate constant of 2.2×10^7 M^{-1} s^{-1}),[28] with CO_3^{2-} (reaction 5) ($\sim 1 \times 10^6$ M^{-1} s^{-1}),[29] or with N_2O (reaction 6) (2.1×10^6 M^{-1} s^{-1}).[30] Under the conditions of our experiments, the observed rate constants for the first two reactions are on the order of 10^3 s^{-1}, whereas those for reactions 5 and 6 are at least 10 times larger. Thus, it is likely that the hydrated electrons produced from reaction 5 react with N_2O (reaction 2) and, together with the $HO^{\bullet}$ generated from reaction 6, contribute to the formation of $CO_3^{\bullet-}$. Reaction of H_2O_2 with the iron(III) and the oxygenated forms of Mb and Hb leads to generation of the high valent oxoiron(IV) form. However, the rate constants for these processes are on the order of 10^2 and 10 M^{-1} s^{-1},[31] respectively, and thus are not relevant in the time scale of the processes discussed in this work.

Protein Solutions. All the solutions used for the pulse radiolysis experiments were prepared by diluting the protein stock solutions (0.5−3 mM) with a N_2O-saturated 0.25 M sodium bicarbonate solution at pH 10.0. In a typical experiment, a 10 mL gastight SampleLock Hamilton syringe was filled with the N_2O-saturated solution. In the same syringe we then added the amount of protein necessary to reach the required concentration (0.2−1.2 mL). To determine the exact concentration, an absorption spectrum of each protein solution was collected with a UVICON 820 spectrophotometer before every experiment. For quantification, we used the extinction coefficients given in ref 32. The concentration of Hb was always expressed per heme. For all proteins studied, we varied the concentration in the range 25−105 μM.

Statistics. At least 5−10 measurements were carried out to determine the observed rate constants at each protein concentrations. The error bars depicted in the figures represent the standard deviation from the mean value. The second-order rate constants were determined from the linear fits of the plots of k_{obs} versus protein concentration and are given as mean values plus or minus the corresponding standard error.

Results and Discussion

Reactions of $CO_3^{\bullet-}$ with Metmyoglobin or Methemoglobin. The reactions of $CO_3^{\bullet-}$ with metMb and metHb were studied by pulse radiolysis at pH 10.0 and room temperature. The two proteins were always present at least in a 6-fold excess to maintain pseudo-first-order conditions. This choice was made to keep the $CO_3^{\bullet-}$ concentration in solution as low as possible, to minimize the fraction of $CO_3^{\bullet-}$ that decays by reacting with itself according to reaction 7 or 8 ($k_{7,8} = 2 \times 10^7$ M^{-1} s^{-1}).[33,34]

$$CO_3^{\bullet-} + CO_3^{\bullet-} \rightarrow CO_2 + CO_4^{2-} \quad (7)$$

$$CO_3^{\bullet-} + CO_3^{\bullet-} \xrightarrow{H_2O} 2CO_2 + HO_2^{-} + HO^{-} \quad (8)$$

For both proteins, the reactions were first studied at different wavelengths on the Soret band (metMb at 411 and 421 nm, and metHb at 410 and 423 nm), but no absorbance changes were detected. This result suggests that $CO_3^{\bullet-}$ does not oxidize the iron(III) center of Mb and Hb, and thus that the corresponding oxoiron(IV) (ferryl) forms of the proteins are not produced.

The reactions of $CO_3^{\bullet-}$ with the amino acid residues of Mb and Hb (reactions 9 and 10) were studied by following the decrease in absorbance at 600 nm, the maximum of the absorbance spectrum of $CO_3^{\bullet-}$ ($\epsilon = 1860 \pm 160$ M^{-1} cm^{-1}).[33]

$$MbFe^{III}OH + CO_3^{\bullet-} \rightarrow {}^{\bullet}MbFe^{III}OH + CO_3^{2-} \quad (9)$$

$$HbFe^{III}OH + CO_3^{\bullet-} \rightarrow {}^{\bullet}HbFe^{III}OH + CO_3^{2-} \quad (10)$$

As shown in Figure 1 (insets), for both metMb and metHb the time courses could be fitted to a single-exponential expression. No further absorbance changes were observed over longer reaction times (up to 9 ms). For both proteins, the observed rate constants (k_{obs}) were linearly dependent on the protein concentrations (Figure 1). The values of the second-order rate constant obtained from the linear fits and given in Table 1 show that the rate constant for the reaction of $CO_3^{\bullet-}$ with metHb is approximately 4 times larger than that for the reaction with metMb. In the concentration range used for the studies with metHb (22−101 μM), the protein was present either as a mixture of dimer and tetramer (22 μM < [metHb] < $\sim$50 μM) or as a tetramer ([metHb] > $\sim$50 μM).[35] As shown in Figure 1B, no deviation from the linearity was observed in the plot of k_{obs} versus the metHb concentration. Thus, the aggregation state of the protein does not influence considerably the reaction rate.

Several amino acids react at a fast rate with the carbonate radical anion, among others, tyrosine (2.9×10^8 M^{-1} s^{-1} at pH 11.2),[36] tryptophan (4.9×10^8 M^{-1} s^{-1} at pH 11.4 and 298 K),[37] methionine (1.2×10^8 M^{-1} s^{-1} at pH 11.4 and 298 K),[37] and cysteine (1.8×10^8 M^{-1} s^{-1} at pH 11.4 and 298 K).[37] The mechanisms of these reactions are different: Oxidation of Tyr has been proposed to take place not via outer-sphere electron transfer but rather via adduct formation.[37] The reaction with Trp has been suggested to occur by addition of $CO_3^{\bullet-}$ to the π

TABLE 1: Summary of the Rate Constants for the Reactions of $CO_3^{\bullet-}$ with Different Forms of Hb and Mb, at pH 10.0 and Room Temperature[a]

reactions	Mb	Hb
$ProteinFe^{III}OH + CO_3^{\bullet-} \rightarrow {}^{\bullet}ProteinFeOH + CO_3^{2-}$	$(4.7 \pm 0.3) \times 10^7\ M^{-1}\ s^{-1}$	$(1.9 \pm 0.3) \times 10^8\ M^{-1}\ s^{-1}$
$ProteinFeO_2 + CO_3^{\bullet-} \rightarrow {}^{\bullet}ProteinFeO_2 + CO_3^{2-}$	$(5.2 \pm 0.3) \times 10^7\ M^{-1}\ s^{-1}$	$(2.1 \pm 0.1) \times 10^8\ M^{-1}\ s^{-1}$
${}^{\bullet}ProteinFeO_2 \rightarrow ProteinFe^{III}OH$	$>6 \times 10^4\ s^{-1}$	$(1.0 \pm 0.2) \times 10^2\ s^{-1}$

[a] The rate constants obtained by assuming an alternative concurring mechanism for the reaction of $CO_3^{\bullet-}$ with oxyMb are given in the text.

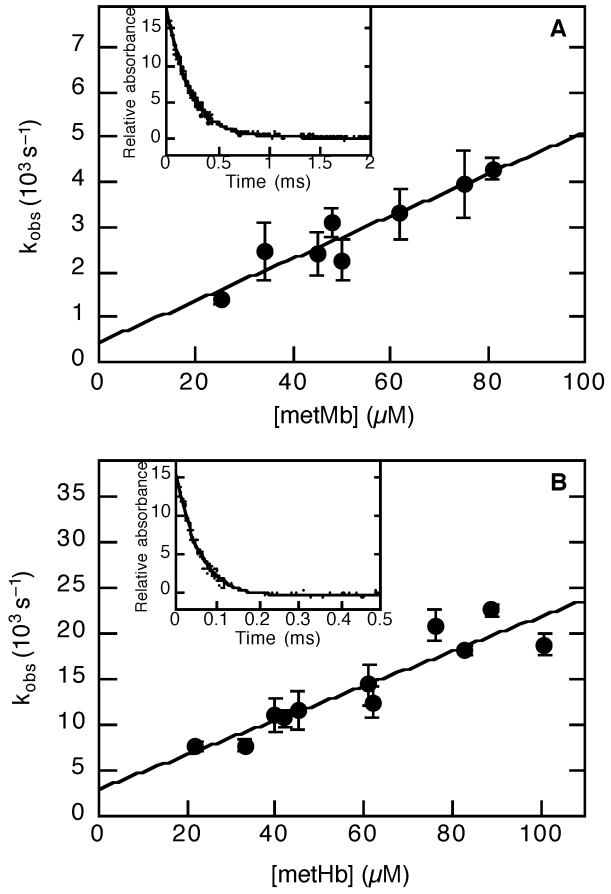

Figure 1. Plots of k_{obs} versus (A) metMb and (B) metHb concentration, for the reaction of $CO_3^{\bullet-}$ with the corresponding protein, at pH 10.0 and room temperature. The second-order rate constants resulting from the linear fits depicted are summarized in Table 1. Insets: time courses measured at 600 nm for the reaction of (A) 81 μM metMb with 10 μM of $CO_3^{\bullet-}$ and of (B) 83 μM metHb with 10 μM $CO_3^{\bullet-}$. Each trace corresponds to the average of 5−10 single traces. The full lines correspond to the best fits to the data resulting in the observed rate constant (A) $k_{obs} = (4.3 \pm 0.2) \times 10^3\ s^{-1}$ and (B) $k_{obs} = (18.2 \pm 0.5) \times 10^3\ s^{-1}$.

system of the indole moiety and not by direct electron transfer.[38] Finally, reaction with Met probably also proceeds via addition to the sulfur, whereas Cys can react by electron transfer from the thiolate group.[37]

In horse heart Mb there are two Tyr, two Trp, and two Met residues but no Cys, whereas in human Hb the α-chain contains three Tyr, one Trp, two Met, and one Cys residues, and the β-chain three Tyr, one Trp, one Met, and two Cys. Because the values of the second-order rate constants for the reactions of $CO_3^{\bullet-}$ with these four amino acids are in the same order of magnitude, $CO_3^{\bullet-}$ is not expected to show any selectivity toward these residues. Thus, the values of the second-order rate constants obtained for the reactions of $CO_3^{\bullet-}$ with metMb and metHb should correspond to the overall rate constants of the reactions of $CO_3^{\bullet-}$ with various amino acid residues on the surface of the two proteins.

To explain the larger rate constant obtained for the reaction with metHb, it is useful to compare the amino acid sequences of the two proteins and the extent of exposure to the solvent of the most reactive residues. Analysis of the three-dimensional structure of Mb shows that Tyr103 and Met55 are located on the surface of the protein and are thus accessible for reaction with $CO_3^{\bullet-}$. In contrast, Trp7 and Trp14 have been reported to be equally exposed to the solvent, but only to an extent of ∼50%.[39] Tyr146 is also only partly accessible. The last Met residue is completely buried in the inside of the globin. Analysis of the structure of the dimeric form of Hb shows that four Tyr residues (Tyrα42, Tyrα140, Tyrβ35, and Tyrβ145) are very well exposed and thus accessible for reaction with $CO_3^{\bullet-}$. The two Trp residues (Trpα14 and Trpβ15), Metβ55, and Cysβ93 are only partly exposed but could also be oxidized by $CO_3^{\bullet-}$. All other reactive amino acids (two Tyr, two Met, and two Cys residues) are not likely to be able to react with externally generated $CO_3^{\bullet-}$. This simple "exposed reactive residues counting" may explain the larger value of the second-order rate constant of the reaction with metHb, which contains a larger number of amino acid residues well accessible for reaction with $CO_3^{\bullet-}$. However, in the tetrameric form of Hb it is more difficult to determine which amino acids are more exposed to the solvent and, thus, with the data obtained up to now it is not possible to ascertain the single contribution of the residues listed above. In conclusion, to unambiguously identify the amino acid residue(s) involved in the reaction with $CO_3^{\bullet-}$, additional experiments are needed; for instance, analogous studies with mutants in which the amino acids more likely to react with $CO_3^{\bullet-}$ are substituted with less reactive residues.

The reaction of metMb (from bovine muscle) with small concentrations of hydroxyl radical has also been shown to proceed without oxidation of the iron center.[24] It has been proposed that $HO^{\bullet}$ reacts with aromatic or heterocyclic groups on the surface of the protein to produce protein radicals expected to react bimolecularly and finally yield dimers and/or trimers, detected by chromatographic analysis of the reaction products.[24] Interestingly, irradiation of metMb solutions with higher doses (up to 500 Gy), to generate significantly larger amounts of $HO^{\bullet}$, leads to a dose-dependent production of both deoxyMb and ferrylMb, up to 20 and 40%, respectively.[25] DeoxyMb has been suggested to be generated by globin radicals through a reductive transfer.[25] In contrast, generation of ferrylMb was ascribed to the reaction of metMb with H_2O_2, an unavoidable product of the radiolysis of aqueous solutions (reaction 1), excluding direct involvement of $HO^{\bullet}$.[25] As mentioned above, reaction of metMb and metHb with $CO_3^{\bullet-}$ does not cause any absorbance changes at various wavelengths on the Soret band, and thus is not likely to cause either oxidation or reduction of the iron(III) center. Taken together, these data suggest that, in contrast to $HO^{\bullet}$, $CO_3^{\bullet-}$ does not generate reducing radicals on the globin and that the concentration of H_2O_2 generated by radiolysis in our solutions was too low to produce detectable amounts of ferrylMb. Interestingly, it has recently been reported that $CO_3^{\bullet-}$ oxidizes Mn(III) porphyrins to their oxoMn(IV) form,[40] whereas nothing

is known on the intrinsic reactivity of $CO_3^{\bullet-}$ toward Fe(III) porphyrin complexes.

Reaction of $CO_3^{\bullet-}$ with Oxyhemoglobin. The reaction of $CO_3^{\bullet-}$ with oxyHb was studied by pulse radiolysis under conditions identical to those used for the reactions with metMb or metHb (pseudo-first-order conditions with oxyHb in excess, pH 10.0, and room temperature). The reaction was first investigated by following the absorbance changes at 541 nm. At this wavelength, we observed a fast decrease followed by a slower further decrease (data not shown). These absorbance changes were assigned to the decay of $CO_3^{\bullet-}$ and to the oxidation of oxyHb to metHb, respectively. $CO_3^{\bullet-}$ has a broad absorbance maximum at 600 nm ($\epsilon = 1860 \pm 160$ M^{-1} cm^{-1})[33] and at 541 nm it still has an extinction coefficient of ~1200 M^{-1} cm^{-1}. Moreover, at 541 nm the ϵ values for oxyHb and metHb (at pH 10.0) are 13.8 and 11.0 mM^{-1} cm^{-1}, respectively.[32] The reaction was also monitored at 430 nm, the isosbestic point of the conversion of oxyHb to metHb and a wavelength at which the extinction coefficient of $CO_3^{\bullet-}$ is below 200 M^{-1} cm^{-1}.[33] As expected, no absorbance changes were observed at this wavelength. Taken together, these data show that the reaction between oxyHb and $CO_3^{\bullet-}$ proceeds in two distinct steps that correspond first to the consumption of $CO_3^{\bullet-}$ and then to the oxidation of the iron center of oxyHb.

The kinetics of the reaction between $CO_3^{\bullet-}$ and oxyHb were studied at 600 nm. As shown in Figure 2, at this wavelength a very fast decrease in absorbance (up to 0.2−0.5 ms), corresponding to the decay of $CO_3^{\bullet-}$, is followed by a slow increase (up to 40 ms), due to the formation of metHb from oxyHb. In a typical experiment, two sets of traces were collected at different time scales (0−2 ms and 0−40 ms) for the same protein concentration. The observed rate constants for the two reaction steps were obtained by first fitting the second reaction step separately to a single-exponential expression, in the range 0.5−40 ms (Figure 2A). As shown in the inset of Figure 2A, the traces collected over 40 ms contained a periodical disturbance of the power line with a frequency of 100 Hz, which could not be eliminated in our experimental setup. Thus, as shown in Figure 2A, the traces were all fitted after first removing this disturbance. The observed rate constant of the first reaction step was then determined by fitting the curve measured for a shorter time to a two-exponential expression in which the value of the second rate constant was fixed to that obtained from the previous fit (Figure 2B). As shown in Figure 2, the fits obtained by using this method reproduce very well the experimental data, both for the short and for the long time scale.

Variation of the oxyHb concentration showed that the observed rate constants for the first reaction step depend linearly on the oxyHb concentration (Figure 3). The value of the second-order rate constant obtained from the linear fit is $(2.1 \pm 0.1) \times 10^8$ M^{-1} s^{-1}, nearly identical to that obtained for the reaction of $CO_3^{\bullet-}$ with metHb (Table 1). In contrast, the observed rate constant for the second reaction step was independent of the protein concentration: the averaged value is $(1.0 \pm 0.2) \times 10^2$ s^{-1}. Taken together, these data suggest that the first reaction step corresponds to the oxidation of amino acid residue(s) of the globin by $CO_3^{\bullet-}$, whereas in the second step the radical(s) generated in the protein oxidize the iron center (reactions 11 and 12).

$$HbFeO_2 + CO_3^{\bullet-} \rightarrow {}^{\bullet}HbFeO_2 + CO_3^{2-} \qquad (11)$$

$${}^{\bullet}HbFeO_2 \rightarrow HbFe^{III}OH \qquad (12)$$

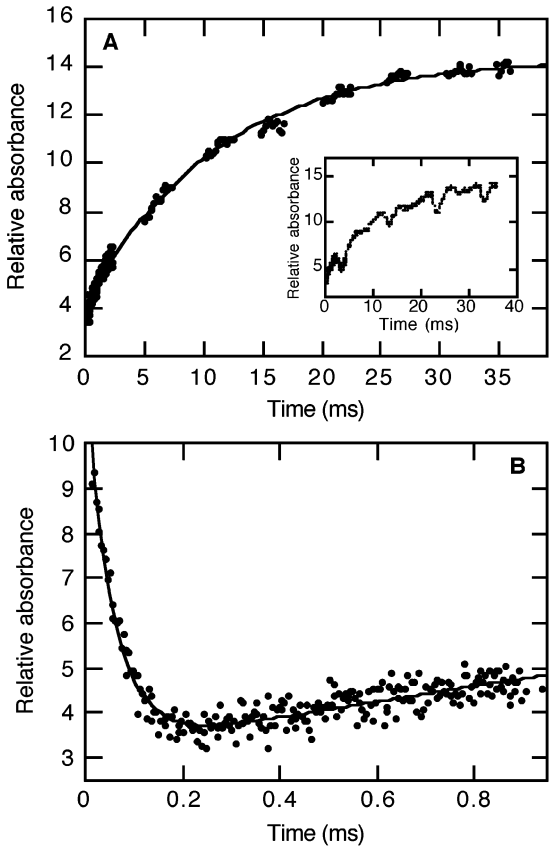

Figure 2. Time courses measured at 600 nm for the reaction of 84 μM oxyHb with 10 μM $CO_3^{\bullet-}$ at pH 10.0 and room temperature: (A) measurement over a longer time scale used to determine the value of k_{obs} of the second reaction step and (B) measurement over a shorter time scale used to determine the value of k_{obs} of the first reaction step. The traces correspond to the average of 7 single traces. The full lines correspond to the best fits to the data resulting in the observed rate constant (A) $k_{obs} = 87 \pm 2$ s^{-1} and (B) $k_{obs} = (17.6 \pm 0.8) \times 10^3$ s^{-1}, for the second and the first reaction steps, respectively. Inset: complete trace measured over a long time scale in which the periodical disturbance, usually cut out before fitting, is clearly visible.

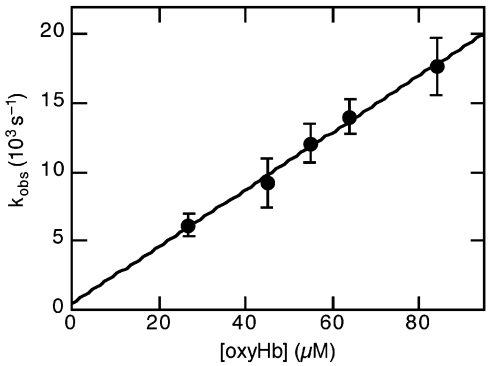

Figure 3. Plot of k_{obs} versus oxyHb concentration for the first step of the reaction of $CO_3^{\bullet-}$ with oxyHb, at pH 10.0 and room temperature. The second-order rate constant resulting from the linear fit depicted is given in Table 1.

Among all the amino acid residues exposed to the solvent, Cysβ93, Tyrα140, Tyrα42, Tyrβ35, and Tyrβ145 are the only ones located maximally ~10 Å from the iron center of the heme and, thus, could oxidize it directly. Nevertheless, it is not possible to exclude other pathways involving hydrogen atom or electron transfers between amino acid residues of the protein before oxidation of the heme. Indeed, it is established that, in the approximate pH range 3.0−12.0, Trp$^{\bullet}$ can transfer its

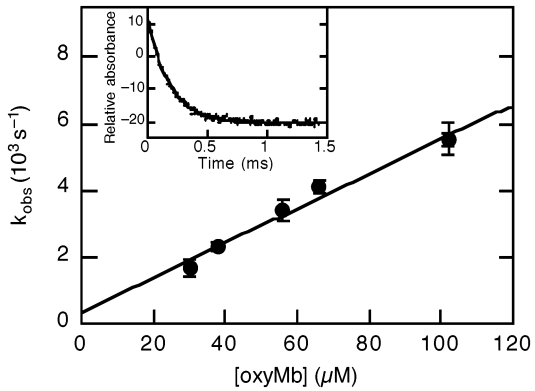

Figure 4. Plot of k_{obs} versus oxyMb concentration for the carbonate radical anion-mediated oxidation of oxyMb, at pH 10.0 and room temperature. The second-order rate constant resulting from the linear fit depicted is given in Table 1. Inset: time course measured at 544 nm for the reaction of 102 μM oxyMb with 10 μM $CO_3^{\bullet-}$. The trace corresponds to the average of 6 single traces. The full line corresponds to the best fit to the data. The resulting rate constant is $k_{obs} = (5.5 \pm 0.2) \times 10^3$ s^{-1}.

electron deficiency to Tyr and thus generate Tyr$^\bullet$. In the Trp-Tyr dipeptide, this process takes place intramolecularly at a rate of about 2×10^5 s^{-1}.[41−43] At pH 7.0, the intermolecular electron-transfer rate between Trp$^\bullet$ and Tyr located on different dipeptides is on the order of 10^5-10^6 M^{-1} s^{-1} [43] and has been proposed to proceed via a proton transfer from Tyr to Trp$^\bullet$, followed by the actual electron-transfer reaction.[44] Moreover, studies with oligoproline-separated Trp and Tyr residues showed that oxidation of Tyr by Trp$^\bullet$ can take place also by long-range electron transfer.[45] Alternatively, the Met/$CO_3^{\bullet-}$ adduct[37] may generate Tyr$^\bullet$ and/or Trp$^\bullet$, in analogy to the Met/$Br_2^{\bullet-}$ adduct.[46]

From the overall absorbance changes at 600 nm, we calculated that, for all protein concentrations, the yield of the carbonate radical anion-mediated oxidation of oxyHb was 36 ± 8% (relative to the $CO_3^{\bullet-}$ concentration). The mechanism discussed above could explain the nonstoichiometric oxidation yields. If Tyr radicals are generated on the surface of the protein, they are expected to react also bimolecularly and thus induce cross-links between subunits, in analogy to the reaction between HO$^\bullet$ and oxyMb.[24,25]

Reaction of $CO_3^{\bullet-}$ with Oxymyoglobin. The reaction of $CO_3^{\bullet-}$ with oxyMb was studied under pseudo-first-order conditions with the protein in excess (at pH 10.0 and room temperature). First, we qualitatively investigated the absorbance changes at different wavelengths. A decrease in absorbance was observed at 430, 440, 544, and 581 nm, whereas an increase was observed at 490 nm. All these spectral changes correspond to those expected for the oxidation of oxyMb to metMb (reaction 13).

$$MbFeO_2 + CO_3^{\bullet-} \rightarrow MbFe^{III}OH + CO_3^{2-} \qquad (13)$$

The kinetics of the oxidation of the iron center were studied at 544 nm, a wavelength at which the absorbance decrease arises mainly from the oxidation of the heme and the contribution from the absorbance of $CO_3^{\bullet-}$ is minimal. As shown in Figure 4, the absorbance changes obeyed first-order kinetics, and the observed rate constants were linearly dependent on the protein concentration. The value of the second-order rate constant obtained from the linear fit is $(5.2 \pm 0.3) \times 10^7$ M^{-1} s^{-1}, very similar to that obtained for the decay of $CO_3^{\bullet-}$ in the presence of metMb (Table 1). The yield of the carbonate radical anion-mediated oxidation of oxyMb was calculated from the absorbance changes at 544 nm. For the protein concentrations in the range 30−66 μM we obtained a value of 46 ± 9% (relative to the $CO_3^{\bullet-}$ concentration). Interestingly, the yields of the reaction of $CO_3^{\bullet-}$ with 102 μM oxyMb were significantly lower ∼30% (relative to the $CO_3^{\bullet-}$ concentration).

Two mechanisms can be formulated to explain these kinetic data. The studies of the reaction of $CO_3^{\bullet-}$ with oxyHb and metHb presented above show that the rate of the reaction of $CO_3^{\bullet-}$ with the amino acid residues of the globin does not depend on the oxidation state of the iron. Thus, in analogy to the reaction with oxyHb, it is conceivable that the reaction of $CO_3^{\bullet-}$ with oxyMb is also a consecutive process in which the first step corresponds to the formation of amino acid radical(s) and the second leads to oxidation of the iron center (reactions 14 and 15):

$$MbFeO_2 + CO_3^{\bullet-} \rightarrow {}^\bullet MbFeO_2 + CO_3^{2-} \qquad (14)$$

$${}^\bullet MbFeO_2 \rightarrow MbFe^{III}OH \qquad (15)$$

However, at all wavelengths studied, the time courses measured for this reaction followed a monoexponential decay. Thus, oxidation of the iron center proceeds at the same rate as the decay of $CO_3^{\bullet-}$. Taken together, these results indicate that the proposed mechanism can explain the experimental data only if oxidation of the globin by $CO_3^{\bullet-}$ (reaction 14) is the rate determining step. This hypothesis would imply that oxidation of the iron center by the amino acid radical(s) takes place at least at a rate of 6×10^4 s^{-1}, a value 10 times larger than the fastest observed rate constant measured. Among the reactive amino acids that are likely to react with $CO_3^{\bullet-}$ (see above), Tyr103 is the residue most exposed to the solvent and closest to the iron center of the heme (the shortest distance between the iron center and a C-atom of the aromatic ring is ∼9.5 Å). The Tyr residue closest to the heme in Hb displays a distance of ∼8.6 Å between the iron center and one of the C-atoms of the ring. Thus, it seems difficult to explain why the rate of iron oxidation in Mb is more than 100 times larger than that in Hb. To get a better understanding of which amino acid residues are involved in these reactions, we are planning to carry out additional experiments with Mb and Hb mutants.

Interestingly, a similar mechanism has been proposed for the reaction of oxyMb (from bovine muscle) with hydroxyl radical, which also yields metMb by intramolecular electron transfer from the metal center to globin radical(s), produced by reaction of HO$^\bullet$ with aromatic groups of the globin moiety.[24] However, in the cited work no information was given on the electron-transfer rate.

An alternative mechanism is based on the hypothesis that two parallel reactions take place: part of $CO_3^{\bullet-}$ may react directly with the iron center and part oxidize the amino acids of the globin (reactions 16 and 17).

$$MbFeO_2 + CO_3^{\bullet-} \rightarrow MbFe^{III}OH + CO_3^{2-} \qquad (16)$$

$$MbFeO_2 + CO_3^{\bullet-} \rightarrow {}^\bullet MbFeO_2 + CO_3^{2-} \qquad (17)$$

If the reaction proceeds according to a concurrent mechanism, the observed rate constant measured at any wavelength must correspond to the sum of the observed rate constants for the two processes ($k_{16,obs} + k_{17,obs}$). This means that the oxidation of oxyMb and the decay of $CO_3^{\bullet-}$ should take place at the same overall rate ($k_{16,obs} + k_{17,obs}$), in agreement with our experimental results. The individual values for k_{16} and k_{17} can be obtained by considering the relative yields of the two processes. As

mentioned above, the oxidation yield of the heme is about 50% and, thus, 50% of $CO_3^{\bullet-}$ should be involved in the production of the radical(s) on the globin. Taken together, these data suggest that, if the reaction proceeds according to this concurrent mechanism, the values of k_{16} and k_{17} should be very similar and correspond to approximately half of the value of the overall second-order rate constant measured ($k_{16} = k_{17} \approx 2.6 \times 10^7$ M^{-1} s^{-1}). In the previous section we have reported a value of $(4.7 \pm 0.7) \times 10^7$ M^{-1} s^{-1} for the second-order rate constant for the reaction of $CO_3^{\bullet-}$ with metMb. As discussed above, in analogy to hemoglobin, the rate constant of the oxidation of the amino acid residues of the globin by $CO_3^{\bullet-}$ is expected to be independent of the oxidation state of the iron. The difference of a factor of 2 obtained between the rate constants of the reactions of $CO_3^{\bullet-}$ with metMb and oxyMb may be within the error of the experiment, but more studies are needed to get a definitive understanding of the mechanism of the latter reaction. Among others, an alternative reaction pathway may be a combination of the two mechanisms just discussed. That is, in addition to the two concurring reactions (reactions 16 and 17), metMb may also be generated by intramolecular electron transfer in $^{\bullet}MbFeO_2$ (reaction 15).

Conclusions and Implications for the Mechanism of the Reactions of Peroxynitrite with oxyMb and oxyHb in the Presence of CO_2. We[16] and others[20] have recently investigated the reaction of peroxynitrite with oxyMb and oxyHb in the presence of 1.2 mM CO_2. A tyrosyl centered radical has been identified by EPR spectroscopy[20] and kinetic studies showed that the reactions proceed in two steps via the formation of the ferryl forms of the proteins.[16] However, simulation of the reaction between oxyMb and an excess of peroxynitrite (in the presence of 1.2 mM CO_2) suggested that an alternative parallel pathway must exist, which generates directly metMb without prior formation of ferrylMb.[16] We have thus postulated that $CO_3^{\bullet-}$, generated from the homolytic cleavage of the O−O bond in $ONOOCO_2^-$, is responsible for such a reaction.[16] The data presented in this work confirm our previous hypothesis. Despite the fact that the yield of $CO_3^{\bullet-}$ produced from $ONOOCO_2^-$ is still a matter of dispute (less than 5%[9] or 30%[7,8]), in the presence of an excess of peroxynitrite $CO_3^{\bullet-}$ is very likely to be scavenged by the protein, and thus lead to direct oxidation of oxyMb and metMb.

Interestingly, we have previously shown that treatment of oxyHb and metHb with an excess of peroxynitrite in the presence of 1.2 mM CO_2 leads to amounts of nitrated Tyr residues larger than those obtained with the corresponding reactions of the Mb forms.[15,17] It is conceivable that this difference is due to the faster rates of the reactions of $CO_3^{\bullet-}$ with the amino acid residues of Hb (in both oxyHb and metHb). These reaction may lead to $Tyr^{\bullet}$ which may rapidly react with $NO_2^{\bullet}$ to generate 3-nitrotyrosine.

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References and Notes

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(13) Abbreviations used: Mb, myoglobin; •Mb, myoglobin with one or more radicals on the globin; metMb, iron(III) myoglobin; ferrylMb, oxoiron(IV) myoglobin; Hb, hemoglobin; •Hb, hemoglobin with one or more radicals on the globin; metHb, iron(III) hemoglobin.
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