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ORIGINAL PAPER

# Oxidation-state-dependent reactions of cytochrome *c* with the trioxidocarbonate(•1–) radical: a pulse radiolysis study

**Anastasia S. Domazou · Willem H. Koppenol**

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**Abstract** The reaction of the trioxidocarbonate(•1–) radical ($CO_3^{\bullet-}$, ''carbonate radical anion'') with cytochrome *c* was studied by pulse radiolysis at alkaline pH and room temperature. With iron(III) cytochrome *c*, $CO_3^{\bullet-}$ reacts with the protein moiety with rate constants of $(5.1 \pm 0.6) \times 10^7$ $M^{-1}$ $s^{-1}$ (pH 8.4, $I \sim 0.27$ M) and $(1.0 \pm 0.2) \times 10^8$ $M^{-1}$ $s^{-1}$ (pH 10, $I = 0.5$ M). The absorption spectrum of the haem moiety was not changed, thus, amino acid radicals produced on the protein do not reduce the haem. The pH-dependent difference in rate constants may be attributed to differences in ionization states of amino acids and to the change in the conformation of the protein. With iron(II) cytochrome *c*, $CO_3^{\bullet-}$ oxidizes the haem quantitatively, presumably via electrostatic guidance of the radical to the solvent-accessible haem edge, with a different pH dependence: at pH 8.4, the rate constant is $(1.1 \pm 0.1) \times 10^9$ $M^{-1}$ $s^{-1}$ and, at pH 10, $(7.6 \pm 0.6) \times 10^8$ $M^{-1}$ $s^{-1}$. We propose that $CO_3^{\bullet-}$ oxidizes the iron center directly, and that the lower rate observed at pH 10 is due to the different charge distribution of iron(II) cytochrome *c*.

**Keywords** Cytochrome *c* · $CO_3^{\bullet-}$ · Pulse radiolysis · Kinetics

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## Introduction

The strongly oxidizing trioxidocarbonate(•1–) radical ($CO_3^{\bullet-}$)[1] reacts rapidly with a number of important biomolecules. Rate constants for its reactions with methionine, cysteine, tryptophan, tyrosine and histidine range from $10^6$ to $10^8$ $M^{-1}$ $s^{-1}$ [3]. Recently, we [4, 5] and others [6] studied the reactions of $CO_3^{\bullet-}$ with haemoglobin and myoglobin. The reaction of $CO_3^{\bullet-}$ with the iron(III) forms of these proteins leads to oxidation of only amino acid residues, with no redox changes observed at the iron centers of the haem, with rate constants on the order of $10^7$–$10^8$ $M^{-1}$ $s^{-1}$. However, during reactions of oxyhaemoglobin and oxymyoglobin with $CO_3^{\bullet-}$, the initially formed amino acid radicals oxidize the iron center.

Nitrosyliron(II) myoglobin and nitrosyliron(II) haemoglobin are also oxidized, with rate constants on the order of $10^8$ $M^{-1}$ $s^{-1}$, to nitrosyliron(III) myoglobin and nitrosyliron(III) haemoglobin, followed by dissociation of nitrogen monoxide. Although the standard electrode potential of $CO_3^{\bullet-}$ is high, $E^\circ\left(CO_3^{\bullet-}/CO_3^{2-}\right) = +1.57 \pm 0.03V$ (Koppenol, Huie, Lymar and Stanbury, 2006, unpublished results), the

[1] Systematic names and trivial or outdated names in parentheses [1, 2]: $CO_3^{\bullet-}$, trioxidocarbonate(•1–) radical, (carbonate radical); $ONOO^-$, oxidoperoxidonitrate anion, (peroxynitrite); $CO_2$, carbon dioxide, dioxidocarbon; $ONOO^-/CO_2$ adduct, 1-carboxylato-2-nitrosodioxidane; $NO^\bullet$, nitrogen monoxide, oxidonitrogen(•), (nitric oxide); $NO_2^\bullet$, nitrogen dioxide, dioxidonitrogen radical; $N_2O$, dinitrogen monoxide, oxidodinitrogen, (nitrous oxide); $O_2^{\bullet-}$, dioxide radical anion, (superoxide); $(SCN)_2^{\bullet-}$, bis(nitridosulfidocarbonate)(*S*–*S*) radical anion, (thiocyanate radical); $N_3^\bullet$, trinitrogen radical, (azide radical); $HCO_3^-$, hydrogen carbonate anion, hydroxidodioxidocarbonate anion; $CO_3^{2-}$, trioxidocarbonate(2–), (carbonate).

radical can be formed in vivo; peroxynitrite, a strong oxidant produced in vivo from the reaction of nitrogen monoxide with superoxide, reacts with carbon dioxide to form an intermediate adduct [7, 8] that decomposes to $CO_3^{\bullet-}$ and nitrogen dioxide with yields of 30% [9, 10] or less than 5% [11]. $CO_3^{\bullet-}$ has also been identified as the product of the oxidation of $HCO_3^-$ or $CO_3^{2-}$ by the strong oxidant formed from the reaction of hydrogen peroxide with copper(II) in copper, zinc superoxide dismutase [12–14]. In addition, formation of $CO_3^{\bullet-}$ during xanthine oxidase turnover has been proposed [15].

Cytochrome *c* is a small haem protein that transfers electrons from cytochrome $c_1$ to cytochrome *c* oxidase in the respiratory chain [16]. The reactions of cytochrome *c* with various radicals generated by pulse radiolysis has been reviewed [17]. Briefly, hydroxyl and hydrogen radicals react with iron(II) cytochrome *c* and iron(III) cytochrome *c* at diffusion-controlled rates to ultimately produce, respectively, partially iron(III) cytochrome *c* and iron(II) cytochrome *c*; the primary targets are amino acid residues, which then undergo redox reactions with the haem [18–22]. $Br_2^{\bullet-}$, $(SCN)_2^{\bullet-}$ and $N_3^{\bullet}$ oxidize iron(II) cytochrome *c* via the solvent-accessible haem edge [20], but attack amino acid residues in iron(III) cytochrome *c* [20]. Cytochrome *c* is also known to react with biologically relevant reducing and oxidizing agents, such as ascorbate [23], superoxide [24, 25] and peroxynitrite [26], but only slowly, if at all, with hydrogen peroxide [27]. Although peroxynitrite readily oxidizes iron(II) cytochrome *c* [26], reduction of iron(III) cytochrome *c* has been observed only in the presence of a large excess of peroxynitrite [28], and is most likely mediated by one-electron oxidation of surface amino acids, which then transfer an electron to the haem. Both reactions are accelerated in the presence of carbon dioxide and involvement of $CO_3^{\bullet-}$ has been proposed [28]. In the same $\gamma$-irradiation study, the reaction of $CO_3^{\bullet-}$ with amino acid residues of iron(III) cytochrome *c* was described; however, no rate constants were reported [28]. The yield of oxidation of iron(II) cytochrome *c* by peroxynitrite is only slightly diminished in the presence of carbon dioxide [29].

In this work, we studied the reactivity of $CO_3^{\bullet-}$ towards both iron(II) cytochrome *c* and iron(III) cytochrome *c* at pH 8.4 and 10 by pulse radiolysis. Our data show that the reaction of $CO_3^{\bullet-}$ is dependent on the oxidation state of iron: the haem center of iron(II) cytochrome *c* is oxidized with a yield of 100%, whereas amino acid residues are the primary target in iron(III) cytochrome *c*.

## Materials and methods

### Materials

All chemicals were of the highest quality available and were used without any further purification. Cytochrome *c* from horse heart was purchased from Sigma (96%, not prepared with trichloroacetic acid). Water was purified with a Millipore Milli-Q unit.

### Cytochrome *c* solutions

Stock solutions of iron(II) cytochrome *c* or iron(III) cytochrome *c* in 0.1 M potassium phosphate pH 6.8, prepared by reducing or oxidizing cytochrome *c* with sodium ascorbate or potassium iron(III) hexacyanoferrate, respectively, and passing the solution through a Sephadex-G-25 column, were kept on ice and used the same day. The cytochrome *c* solutions used for the pulse radiolysis experiments were prepared by adding appropriate amounts of stock solutions to 0.25 M sodium hydrogen carbonate solutions saturated with dinitrogen monoxide, at pH 8.4 or 10. The pH was adjusted by addition of sodium hydroxide. The protein concentration was determined spectrophotometrically at 550 nm from the absorbance difference between reduced and oxidized cytochrome *c* with $\Delta\varepsilon = 21{,}100\ M^{-1}\ cm^{-1}$ [30].

### Pulse radiolysis experiments

For the pulse radiolysis experiments, a Febetron 705 2.3-MeV electron accelerator (Titan Systems) with a pulse width of less than 50 μs was used. The light source was a 75-W xenon arc lamp and the optical path length of the quartz cell (Hellma) was 1 or 2 cm. The detection system consisted of an Acton SP300 monochromator (Roper Scientific) and a R928 photomultiplier (Hamamatsu), a DHPCA-200 amplifier (Femto Messtechnik) and a DL7100 digital storage oscilloscope (Yokogawa Electric Corporation). The dose per pulse used was 4–16 Gy and was determined with a thiocyanate dosimeter [31]. $CO_3^{\bullet-}$ species were generated upon irradiation of aqueous solutions of 0.25 M sodium hydrogen carbonate, saturated with dinitrogen monoxide (24.4 mM), at pH 8.4 or 10 at room temperature at concentrations of 1.5–10 μM, depending on the pH and the protein concentration.

The error bars in the figures were drawn to reflect the confidence intervals for 95% probability. The second-order rate constants were derived from the linear plot of $k_{obs}$ versus protein concentration and are given as (mean value ± $t_s s$).

## Results

### Generation of $CO_3^{\bullet-}$ by pulse radiolysis in the presence of cytochrome *c*

Under the conditions used in the pulse radiolysis experiments, hydrated electrons, hydroxyl radicals and most of the hydrogen atoms are converted to $CO_3^{\bullet-}$ via the reactions in Eqs. 1, 2, 3, 4, 5 and 6:

$$e_{aq}^- + N_2O + H_2O \rightarrow {}^{\bullet}OH + N_2 + OH^-$$

$$k_1 = 9.1 \times 10^9\ M^{-1}\ s^{-1}\ [32], \quad (1)$$

$${}^{\bullet}OH + HCO_3^- \rightarrow CO_3^{\bullet-} + H_2O$$

$$k_2 = 8.5 \times 10^6\ M^{-1}\ s^{-1}\ [33] \quad (2)$$

$${}^{\bullet}OH + CO_3^{2-} \rightarrow CO_3^{\bullet-} + OH^-$$

$$k_3 = 4.2 \times 10^8\ M^{-1}\ s^{-1}\ [33] \quad (3)$$

$$H^{\bullet} + CO_3^{2-} \rightarrow HCO_3^- + e_{aq}^-$$

$$k_4 = \text{ca.}1 \times 10^6\ M^{-1}\ s^{-1}\ [34] \quad (4)$$

$$H^{\bullet} + HCO_3^- \rightarrow \text{interm. adduct}$$

$$k_5 = 4.4 \times 10^4\ M^{-1}\ s^{-1}\ [35] \quad (5)$$

$$H^{\bullet} + N_2O \rightarrow N_2 + {}^{\bullet}OH$$

$$k_6 = 2.1 \times 10^6\ M^{-1}\ s^{-1}\ [36] \quad (6)$$

In the presence of iron(II) cytochrome *c* or iron(III) cytochrome *c*, reactions of hydroxyl and hydrogen radicals with the protein compete with the reactions in Eqs. 2, 3, 4, 5 and 6: hydroxyl and hydrogen radicals react with iron(III) cytochrome *c* with rate constants of $1.4 \times 10^{10}\ M^{-1}\ s^{-1}$ [22] and $1.1 \times 10^{10}\ M^{-1}\ s^{-1}$ [18, 19], respectively, to give, among other products, 25–55% iron(II) cytochrome *c* [21] and 50% iron(II) cytochrome *c* [18, 19], respectively. The corresponding rate constants for the reactions of these radicals with iron(II) cytochrome *c* are higher than $1 \times 10^{10}\ M^{-1}\ s^{-1}$ [20] and $1.3 \times 10^{10}\ M^{-1}\ s^{-1}$, respectively [19]. Only 5% of all hydroxyl radicals oxidize the haem [20].

At both pH values and depending on the protein concentration, a fraction of hydrogen radicals react with the oxidized or reduced form of cytochrome *c*. The remainder, aside from those consumed in the reaction in Eq. 5, are converted to hydroxyl radicals via the reactions in Eqs. 1, 4 and 6, and thus contribute to the formation of $CO_3^{\bullet-}$ through the reactions in Eqs. 2 and 3.

At pH 10, where the concentrations of $HCO_3^-$ and $CO_3^{2-}$ are 0.10 and 0.15 M, respectively ($pK_a$ 9.8 at $I = 0.5$ M [37]), and for the protein concentrations used (up to 100 μM), hydroxyl radicals do not react with cytochrome *c* but are completely converted to $CO_3^{\bullet-}$. At pH 8.4 and with the same $pK_a$ value, we calculate concentrations of $HCO_3^-$ and $CO_3^{2-}$ as 0.241 and 0.009 M, respectively. Given the low rate constant for the reaction of the hydroxyl radical with $HCO_3^-$, a fraction of the hydroxyl radicals react with cytochrome *c*. Because the $pK_a$ does not vary substantially over the ionic strength range 0.1–0.5 M ($pK_a$ 9.93 at $I = 0.1$ M compared with 9.8 at $I = 0.5$ M [37]), we used the $pK_a$ value of 9.8 for calculations of the ionic strength at both pH 8.4 and 10. We calculated the *G* values of hydroxyl and hydrogen radicals that react with cytochrome *c* as well as the *G* values of $CO_3^{\bullet-}$ formed in the presence of various concentrations of cytochrome *c* at pH 8.4. In these calculations, the fraction of hydrogen radicals converted to hydroxyl radicals was also taken into account. From the *G* values, the corresponding concentrations of radicals were calculated for a total dose of 8 Gy and for both forms of cytochrome *c* (Fig. 1). For calculations involving iron(II) cytochrome *c*, the rate constant for reaction with hydroxyl radicals was assumed to be $2 \times 10^{10}\ M^{-1}\ s^{-1}$, since no experimentally determined value has been reported.

### $CO_3^{\bullet-}$ and iron(III) cytochrome *c*

The reaction of $CO_3^{\bullet-}$ with iron(III) cytochrome *c* was studied by pulse radiolysis with a dose of 5–12 Gy at pH 8.4 and 10. The concentration of $CO_3^{\bullet-}$ was maintained at less than 6 μM, and at least fivefold excess protein was always present to avoid recombination of the $CO_3^{\bullet-}$ radicals ($k_7 = 2 \times 10^7\ M^{-1}\ s^{-1}$ [38, 39]) and to ensure pseudo-first-order conditions. At wavelengths below 450 nm, only minor absorbance changes were observed. Absorbance changes observed at wavelengths above 450 nm are due only to $CO_3^{\bullet-}$, which has a broad absorption band centered at 600 nm ($\varepsilon = 1{,}860\ M^{-1}\ cm^{-1}$ [39]). After the accelerated decay of $CO_3^{\bullet-}$ in the presence of iron(III) cytochrome *c*, a barely detectable absorbance increase at 550 nm was observed, corresponding to reduction of the haem.

The kinetics of the reaction of $CO_3^{\bullet-}$ with the iron(III) cytochrome *c* was studied mainly at 600 nm; kinetics data collected at other wavelengths gave results identical to those at 600 nm and were also used to determine rate constants. The decay of $CO_3^{\bullet-}$ at all wavelengths studied and under both conditions of pH can be fit to a single-exponential (Fig. 2). For concentrations of iron(III) cytochrome *c* below 20 μM, the

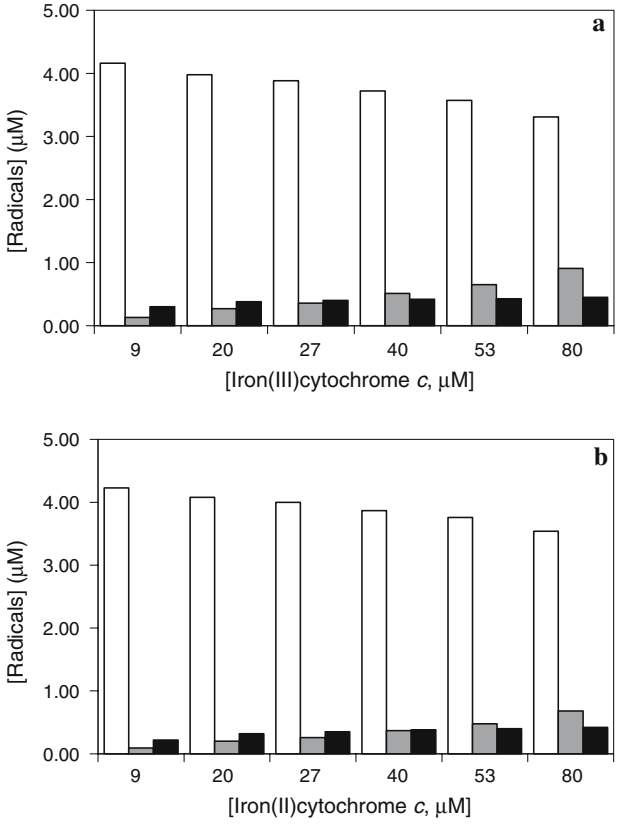


**Fig. 1** At higher concentrations of cytochrome *c*, the protein scavenges partially hydroxyl and hydrogen radicals that thus cannot be converted to $CO_3^{\bullet-}$: calculated concentrations of $CO_3^{\bullet-}$ formed (*white bars*) and of hydroxyl radicals (*grey bars*) and hydrogen radicals (*black bars*) that react with iron(III) cytochrome *c* and iron(II) cytochrome *c*, at various concentrations of iron(III) cytochrome *c* (**a**) and iron(II) cytochrome *c* (**b**), in the presence of 0.25 M $HCO_3^-/CO_3^{2-}$, 24.4 mM dinitrogen monoxide, at pH 8.4, and at a dose of 8 Gy

bimolecular decay of $CO_3^{\bullet-}$ with itself was taken into account. At each pH, the first-order rate constants measured are linearly dependent on the concentration of iron(III) cytochrome *c* (Fig. 3); second-order rate constants of $(5.1 \pm 0.6) \times 10^7$ $M^{-1}$ $s^{-1}$ at pH 8.4 and $(1.0 \pm 0.2) \times 10^8$ $M^{-1}$ $s^{-1}$ at pH 10 were derived (Table 1).

At pH 10, all hydroxyl radicals are converted to $CO_3^{\bullet-}$: the concentrations of $CO_3^{\bullet-}$ on the basis of the absorbance at 600 nm indicate, within experimental error, a yield of 100% (relative to hydroxyl radicals). At pH 8.4, a fraction of the hydroxyl radicals are not scavenged by $HCO_3^{\bullet-}/CO_3^{\bullet-}$, but react directly with iron(III) cytochrome *c*. The theoretically expected concentrations of $CO_3^{\bullet-}$ at various iron(III) cytochrome *c* concentrations (Fig. 1) are in good agreement with those determined experimentally.

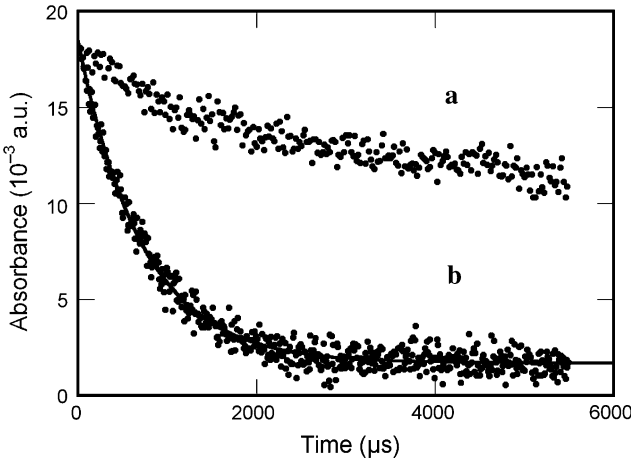


**Fig. 2** Decay of $CO_3^{\bullet-}$ in the absence (*curve a*) and presence (*curve b*) of iron(III) cytochrome *c*. *Curve b*: solutions of 26 μM cytochrome *c* containing 0.25 M $HCO_3^-/CO_3^{2-}$, saturated with dinitrogen monoxide, were irradiated (4.6 μM $CO_3^{\bullet-}$); absorbance changes at pH 8.4 and room temperature at 600 nm are shown as a function of time; optical path length 2 cm. The single-exponential fit is also shown. *Curve a*: same conditions as described for *curve b*, but without iron(III) cytochrome *c*

## $CO_3^{\bullet-}$ and iron(II) cytochrome *c*

The reaction of $CO_3^{\bullet-}$ with iron(II) cytochrome *c* was studied under exactly the same conditions as those used for iron(III) cytochrome *c*. The absorbance changes recorded in the range 340–600 nm correspond to the difference spectrum of iron(II) cytochrome *c* and iron(III) cytochrome *c* (Fig. 4). These data are consistent with oxidation of iron(II) cytochrome *c* to iron(III) cytochrome *c*.

The formation of iron(III) cytochrome *c*—followed at wavelengths below 480 nm, where the absorption of $CO_3^{\bullet-}$ is negligible—and the decay of $CO_3^{\bullet-}$—followed at the isosbestic points for the conversion between iron(II) cytochrome *c* and iron(III) cytochrome *c*—are synchronous (Fig. 5). At pH 10, after the complete decay of $CO_3^{\bullet-}$, a slow absorbance increase in the range 340–400 nm is observed (Fig. 6), whereas the absorbance in the range 400–600 nm remains unchanged. This slow process, which is complete within approximately 100 ms, is not observed at pH 8.4.

Rate curves in the range 340–600 nm were fit to a single-exponential and yielded identical rate constants. The kinetics of the reaction of iron(II) cytochrome *c* with $CO_3^{\bullet-}$ were studied mainly at 556 nm, one of the isosbestic points of iron(II) cytochrome *c* and iron(III) cytochrome *c*; however, values from other wavelengths were also used. At each pH, the observed first-order rate constants are linearly dependent on the protein concentration (Fig. 7), and second-order rate constants

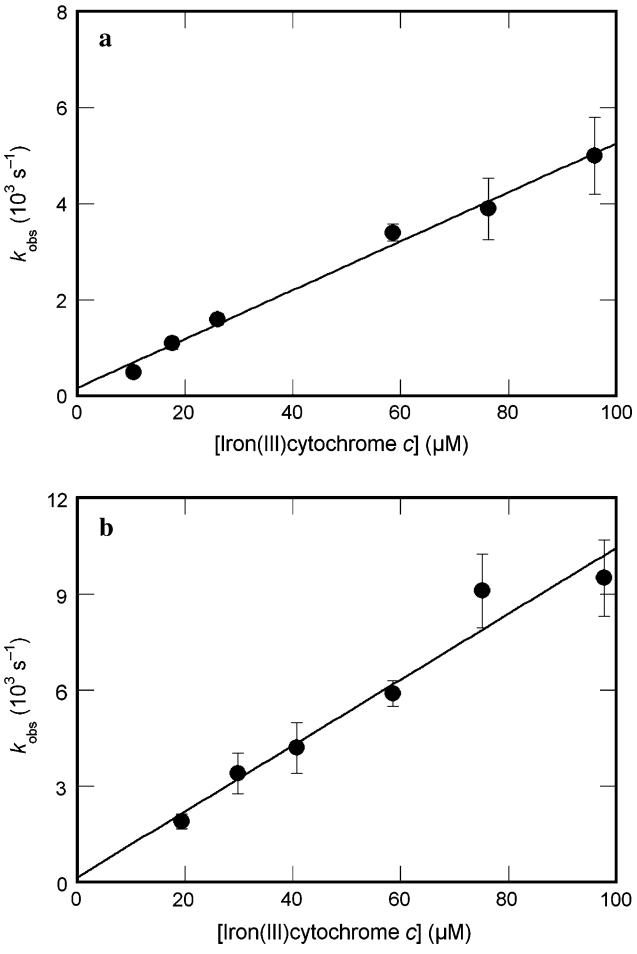


**Fig. 3** Dependence on protein concentration of the apparent first-order rate constant, $k_{obs}$, for the reaction of $CO_3^{\bullet-}$ with iron(III) cytochrome *c* at room temperature at pH 8.4 (**a**) and pH 10 (**b**)

**Table 1** Rate constants of the reaction of $CO_3^{\bullet-}$ with iron(II) cytochrome *c* and iron(III) cytochrome *c* at room temperature, and pH 8.4 and 10

| Reaction | Rate ($M^{-1}$ $s^{-1}$) | |
|---|---|---|
| | pH 8.4 | pH 10 |
| Fe(III) cytochrome *c* + $CO_3^{\bullet-}$ | $(5.1 \pm 0.6) \times 10^7$ | $(1.0 \pm 0.2) \times 10^8$ |
| Fe(II) cytochrome *c* + $CO_3^{\bullet-}$ | $(1.1 \pm 0.1) \times 10^9$ | $(7.6 \pm 0.6) \times 10^8$ |

of $(1.1 \pm 0.1) \times 10^9$ $M^{-1}$ $s^{-1}$ at pH 8.4 and $(7.6 \pm 0.6) \times 10^8$ $M^{-1}$ $s^{-1}$ at pH 10 were derived (Table 1).

The slow process observed at 340–400 nm is a first-order process with a rate constant of $50 \pm 10$ $s^{-1}$ that is independent of the protein concentration. The large error in the rate constant is due to small absorption changes at the detection limits of the instruments.

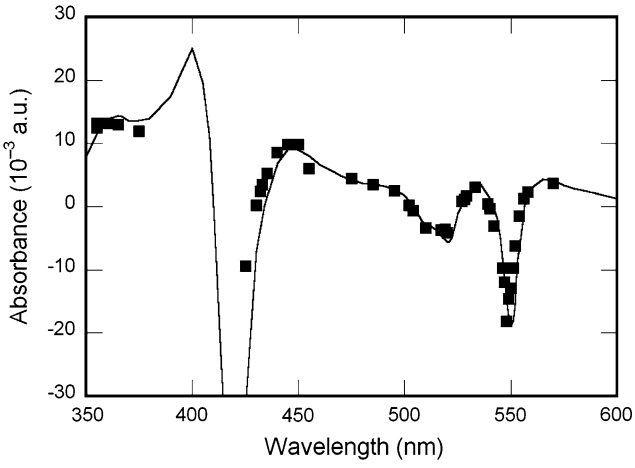


**Fig. 4** Difference spectrum of the reaction of $CO_3^{\bullet-}$ with iron(II) cytochrome *c*. Solutions of 18 μM cytochrome *c* containing 0.25 M $HCO_3^-/CO_3^{2-}$, saturated with dinitrogen monoxide, were irradiated (4 μM $CO_3^{\bullet-}$); absorbance changes were measured 2,000 μs after the pulse at pH 8.4 and room temperature; optical path length 2 cm; normalized dose 1 Gy. The experimental points shown fit a line calculated from data in [50]

As with iron(III) cytochrome *c*, at pH 10, all radicals except for a fraction of the hydrogen atoms are quantitatively converted to $CO_3^{\bullet-}$, while at pH 8.4, a fraction of the hydroxyl radicals react directly with iron(II) cytochrome *c*. The theoretically expected (Fig. 1) and measured concentrations of $CO_3^{\bullet-}$ at different iron(II) cytochrome *c* concentrations are in good agreement. At both pH values, the yield of iron(III) cytochrome *c* from oxidation of iron(II) cytochrome *c*, measured at 480 and 550 nm, is 100%, relative to the initial $CO_3^{\bullet-}$ concentration.

## Discussion

The results show that $CO_3^{\bullet-}$ reacts with both reduced and oxidized forms of cytochrome *c* with rate constants that differ by a factor of about 10. Reactions of hydroxyl and hydrogen radicals with the protein also occur but do not interfere with the interpretation of the kinetics data, which were derived from the decay of $CO_3^{\bullet-}$ under conditions in which the protein was always present in excess.

### $CO_3^{\bullet-}$ and iron(III) cytochrome *c*

$CO_3^{\bullet-}$ reacts with iron(III) cytochrome *c* via a simple second-order reaction, and the absorbance changes recorded corresponded only to the formation and decay of $CO_3^{\bullet-}$. Given the absence of absorbance

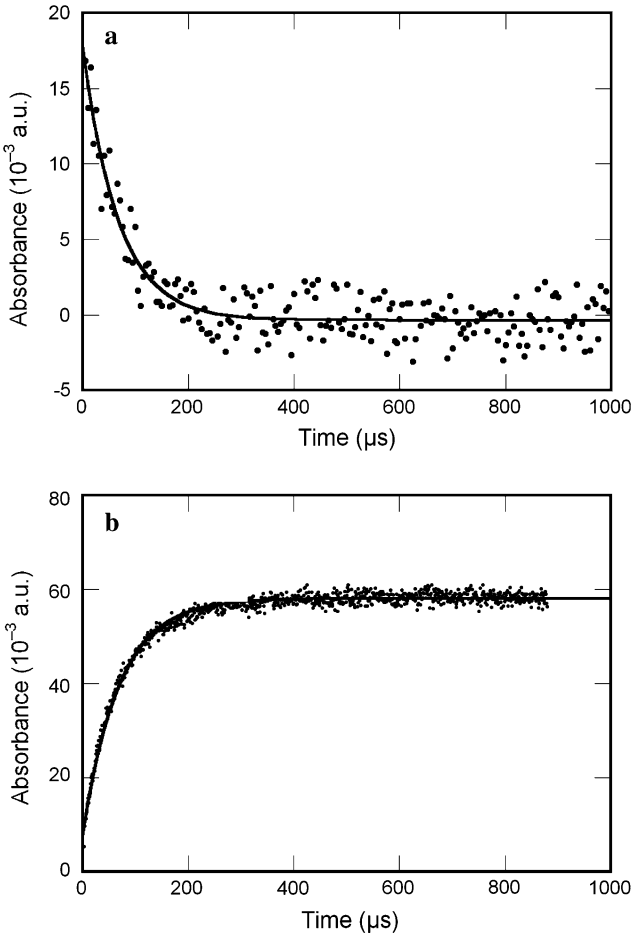


**Fig. 5** Reaction of $CO_3^{\bullet-}$ with iron(II) cytochrome *c* at pH 10 and room temperature. Solutions of 32.5 μM cytochrome *c* containing 0.25 M $HCO_3^-/CO_3^{2-}$, saturated with dinitrogen monoxide, were irradiated (4.5 μM $CO_3^{\bullet-}$); optical path length 2 cm. The reduction of $CO_3^{\bullet-}$ was followed at 556 nm (**a**) and the oxidation of iron(II) cytochrome *c* at 480 nm (**b**); the initial absorbance at 480 nm is due to the $CO_3^{\bullet-}$. The single-exponential fit is also shown

changes in the region of the α and β bands of cytochrome *c*, we conclude that $CO_3^{\bullet-}$ reacts only with amino acid residues of the protein, and that the amino acid radicals formed do not significantly influence the oxidation state of the haem:

$$\mathrm{Fe(III)cyt}\,c(\mathrm{amino\,acids})_x + \mathrm{CO_3^{\bullet-}} \rightarrow \mathrm{Fe(III)cyt}\,c(\mathrm{amino\,acids})_x^{\bullet} + \mathrm{CO_3^{2-}} \quad (7)$$

The delayed and small absorbance increase detected at 550 nm is likely due to reduction of iron(III) cytochrome *c* by hydroxyl and hydrogen radicals rather than by amino acid radicals formed via the reaction in Eq. 7. Indeed, at high iron(III) cytochrome *c* concentrations, a nonnegligible fraction of hydroxyl and hydrogen radicals react within 100 ms to give iron(II)

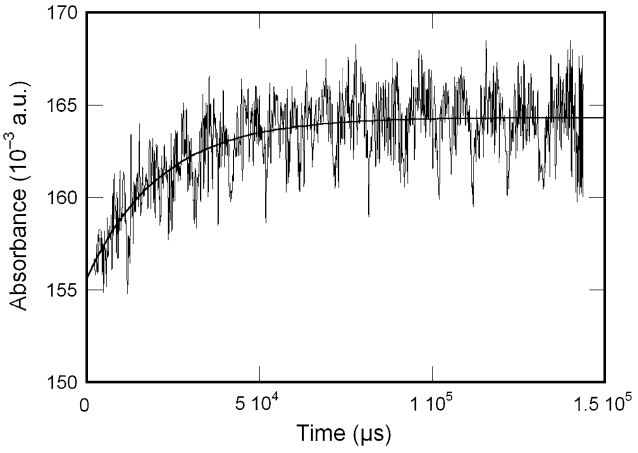


**Fig. 6** Slow process observed after the reaction of $CO_3^{\bullet-}$ with iron(II) cytochrome *c* at pH 10. Solutions of 32.5 μM cytochrome *c* containing 0.25 M $HCO_3^-/CO_3^{2-}$, saturated with dinitrogen monoxide, were irradiated (4 μM $CO_3^{\bullet-}$) at room temperature; optical path length 2 cm; absorbance changes at 375 nm recorded as a function of time. The single-exponential fit is also shown

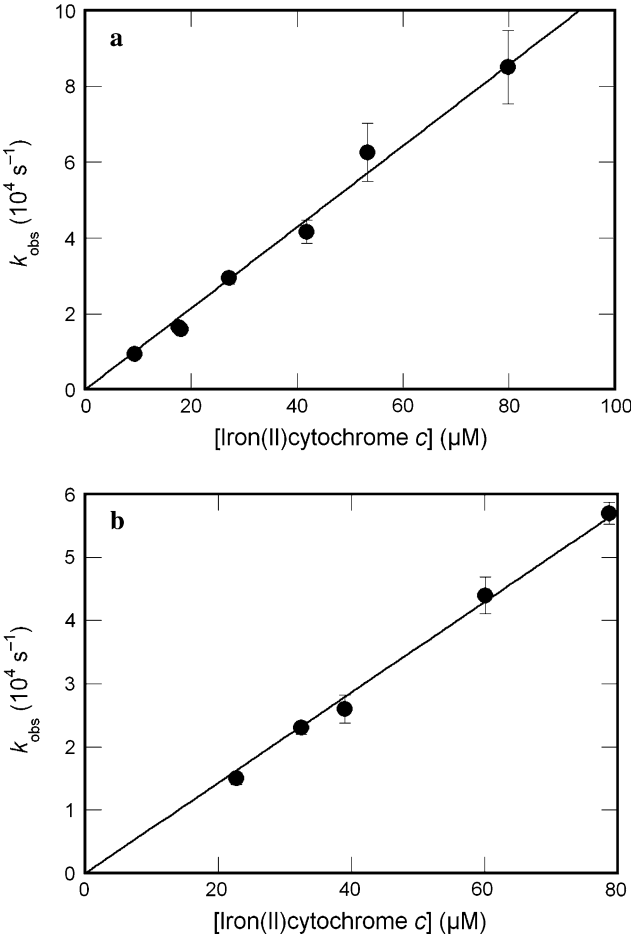


**Fig. 7** Dependence on protein concentration of the apparent first-order rate constant, $k_{obs}$, for the reaction of $CO_3^{\bullet-}$ with iron(II) cytochrome *c* at room temperature at pH 8.4 (**a**) and pH 10 (**b**)

cytochrome *c* with 20–50 and 50% yields, respectively [19, 21]. At pH 8.4, 50 μM protein, and a dose of 8 Gy, this would correspond to formation of 0.2–0.5 μM iron(II) cytochrome *c* and an absorbance increase of 0.004–0.01 absorbance units at 550 nm (which compares quite well with the measured values of 0.002–0.007). Alternatively, if the reduction is caused by electron transfer from certain amino acid radicals (see later) caused by $CO_3^{\bullet-}$, then the yield of intramolecular haem reduction is 1–4%. Thus, these radicals do not significantly reduce the haem of iron(III) cytochrome *c*. Similar results have been reported for the reaction of $CO_3^{\bullet-}$ with methaemoglobin and metmyoglobin [4] and for the reactions of iron(III) cytochrome *c* with $Br_2$, $(SCN)_2^{\bullet-}$ and $N_3^{\bullet}$ [20].

Since $CO_3^{\bullet-}$ reacts much more rapidly with cysteine, tyrosine, tryptophan, histidine and methionine than with other amino acids, with rates on the order of $10^6$–$10^8$ $M^{-1}$ $s^{-1}$ [3, 40], these are the amino acids in cytochrome *c* regarded as targets for $CO_3^{\bullet-}$. The reactions of $CO_3^{\bullet-}$ with tyrosine, histidine and tryptophan involve attack on the aromatic ring; with methionine and cysteine, attack occurs at the sulphur atom. Cytochrome *c* from horse heart has four tyrosine, one tryptophan, three histidine, two methionine and two haem-linked cysteine residues [41]. Of these, two tyrosines, two histidines, one methionine and one cysteine haem linkage are at least partially exposed to the solvent [42, 43]. At pH 10, the hydroxy group of tyrosine is deprotonated, which could be responsible for the observed difference in the rate constants for the reaction in Eq. 7 at pH 10 and 8.4. It is known that, at pH 9.0–9.5, iron(III) cytochrome *c* from horse heart adopts a more open conformation [44, 45], and this more open conformation of iron(III) cytochrome *c* may also contribute to the higher rate constant at pH 10.

## $CO_3^{\bullet-}$ and iron(II) cytochrome *c*

$CO_3^{\bullet-}$ oxidizes iron(II) cytochrome *c* with quantitative formation of iron(III) cytochrome *c*. The decay of $CO_3^{\bullet-}$ and the formation of iron(III) cytochrome *c* take place at the same rate, and the calculated second-order rate constants at pH 8.4 and 10 are 1 order of magnitude higher than those of the reaction of $CO_3^{\bullet-}$ with iron(III) cytochrome *c*. Moreover, the rate constants for iron(II) cytochrome *c* and iron(III) cytochrome *c* depend on pH in opposite ways. These data also suggest that a mechanism involving reaction of $CO_3^{\bullet-}$ only with amino acid residues that in turn oxidize the iron center can be ruled out. In analogy to the reactions of hydrated electrons, $CO_2^{\bullet-}$, superoxide, $Br_2^{\bullet-}$, $(SCN)_2^{\bullet-}$ and $N_3^{\bullet}$, we propose that $CO_3^{\bullet-}$ reacts with iron(II) cytochrome *c* at the solvent-accessible haem edge. This area occupies approximately 1% of the surface of cytochrome *c*, is more easily oxidized than an amino acid, and negatively charged reactants are guided towards it by the electric potential field generated by the asymmetric charge distribution of the protein [46, 47].

$$\text{Fe(II)cyt}\,c + CO_3^{\bullet-} \rightarrow \text{Fe(III)cyt}\,c + CO_3^{2-} \qquad (8)$$

Given that the oxidation of iron(II) cytochrome *c* at all wavelengths studied was synchronous with the decay of $CO_3^{\bullet-}$, we exclude the possibility that the reaction in Eq. 8 takes place in parallel to a process in which an amino acid is oxidized, followed by intramolecular electron transfer from the haem to that amino acid. Thus, the rate constant of the reaction in Eq. 8 is $(1.1 \pm 0.1) \times 10^9$ $M^{-1}$ $s^{-1}$ at pH 8.4, which is higher that the one at pH 10 of $(7.6 \pm 0.6) \times 10^8$ $M^{-1}$ $s^{-1}$. Similar observations have been reported for the reaction of the hydrated electrons with iron(II) cytochrome *c* [17, 48]. Since iron(II) cytochrome *c* does not undergo any transition to an alkaline conformation in the pH range 8–10 [45], the authors have attributed the lower value at alkaline pH to the smaller positive net charge of cytochrome *c*, which may also apply here. We note that at pH 10 the ionic strength is higher ($I$ = 0.5 M) than at pH 8.4 ($I$ ~ 0.27 M) and that this also contributes to the rate constant being smaller. The reactions of $Br_2^{\bullet-}$, $(SCN)_2^{\bullet-}$ and $N_3^{\bullet}$ with iron(II) cytochrome *c* appear to proceed by the same mechanism with reactivities and efficiencies similar to those of $CO_3^{\bullet-}$, but the corresponding rate constants do not vary over the pH range from pH 7 to 8.9. However, the measurements with $CO_3^{\bullet-}$ were carried out at pH 8.4 and 10.

Interestingly, $CO_3^{\bullet-}$ oxidizes oxyhaemoglobin and oxymyoglobin by a different mechanism [4, 6]. $CO_3^{\bullet-}$ reacts initially with the globin with rate constants at pH 10 of $(5.2 \pm 0.3) \times 10^7$ $M^{-1}$ $s^{-1}$ for oxymyoglobin [4] and $(2.1 \pm 0.1) \times 10^8$ $M^{-1}$ $s^{-1}$ for oxyhaemoglobin [4]. The globin radicals then oxidize the iron center in a slow first-order process (100 $s^{-1}$ [4] or 23 $s^{-1}$ [6] for oxyhaemoglobin). In an alternative mechanism proposed for oxymyoglobin, $CO_3^{\bullet-}$ oxidizes directly both 50% of the iron center and amino acids concurrently [4].

The slow process occurring at pH 10 that lasts about 100 ms with an observed absorbance increase in the

range 370–400 nm (rate constant, 50 ± 10 $s^{-1}$) cannot be attributed to oxidation of iron(III) in cytochrome *c* as it is not accompanied by a simultaneous decrease in absorbance at 550 nm. This process is more likely a conformational change of iron(III) cytochrome *c* to the more open alkaline conformation. A comparable rate (10 $s^{-1}$) was reported for the conformational change from the reduced conformation to the oxidized conformation of iron(III) cytochrome *c* at pH 7 by circular dichroism spectroscopy [49].

In conclusion, the present results show that cytochrome *c* reacts rapidly with $CO_3^{\bullet-}$. The enzymatically active reduced form of the protein is preferentially attacked, and the haem is exclusively oxidized with 100% efficiency. Amino acid residues, i.e., tyrosine, tryptophan, methionine, histidine and cysteine, are vulnerable to oxidation by $CO_3^{\bullet-}$ only when the haem is already oxidized, and this reaction becomes important only when the iron(III) cytochrome *c* concentration is much higher than that of iron(II) cytochrome *c*.

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