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The reaction of ascorbic acid with different heme iron redox states of myoglobin

Antioxidant and prooxidant aspects

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The interaction of ascorbate with different heme iron redox states of myoglobin (ferrylmyoglobin, $Fe^{IV}=O$; metmyoglobin, Fe^{III}; and oxymyoglobin, $Fe^{II}O_2$) was examined by e.s.r. and absorption spectroscopy. The reaction of ascorbate with ferryl- or met-myoglobin resulted in ascorbyl radical production. The interaction of ascorbate with oxymyoglobin proceeded with formation of ascorbyl radical, hydrogen peroxide, and an overall oxidation of oxymyoglobin to metmyoglobin. The latter reaction proceeded via an oxoferryl complex intermediate – corresponding to ferrylmyoglobin and identified by treatment of the reaction mixture with Na_2S. These observations are consistent with a concerted electron transfer mechanism, whereby the two electrons required for the reduction of oxygen to hydrogen peroxide are donated by ascorbic acid and the heme iron. The antioxidant and prooxidant aspects of these redox transitions are discussed in terms of their kinetic properties.

Ascorbate; Ascorbyl radical; Ferrylmyoglobin; Oxymyoglobin; Antioxidant

1. INTRODUCTION

The oxidant generated during the reaction of metmyoglobin (or methemoglobin) with H_2O_2 is known as ferrylmyoglobin (or ferrylhemoglobin), a species which contains hypervalent heme iron in the form of an oxoferryl complex as well as an amino acid radical [1,2]. The heterolytic cleavage of the O–O bond of the peroxide by myoglobin leading to ferrylmyoglobin formation is conventionally written as in reaction 1 (where $Fe^{IV}=O$, stands for the oxoferryl moiety in ferrylmyoglobin; HX- for an amino acid; Fe^{III} for metmyoglobin).

$$HX\text{-}Fe^{III} + H_2O_2 \rightarrow {}^{\bullet}X\text{-}Fe^{IV}=O + H_2O \quad \textbf{(1)}$$

Ferrylmyoglobin has assumed high significance within the context of coronary heart diseases [3–5], a notion partly based on the broad chemical reactivity and long-life time of the oxoferryl complex and, hence, its potential implication in cellular oxidative damage. As a corollary, several antioxidants or electron donors have been evaluated in terms of their ability to reduce the hypervalent heme iron in ferrylmyoglobin to met- or oxymyoglobin [6–8]. Ascorbic acid reduces efficiently ferrylmyoglobin [8–10] and also metmyoglobin [10] – albeit at slower rates. Although absorption spectral evidence was provided for the ferrylmyoglobin → metmyoglobin redox transition [8–10], the generation of ascorbyl radical ($A^{\bullet -}$) by this process remained hypothetical. The formation of this weak oxidizing radical is expected to ensue from the electron-transfer depicted in reaction **(2)**.

$$HX\text{-}Fe^{IV}=O + AH^- + H^+ \rightarrow HX\text{-}Fe^{III} + A^{\bullet -} + H_2O \quad \textbf{(2)}$$

In this study, we provide evidence that the formation of ascorbyl radical is a feature inherent not only in the reaction of ascorbate with the hypervalent heme iron, but also in the reaction with the ferric (metmyoglobin) and ferrous (oxymyoglobin) forms of the hemoprotein. The interaction of ascorbate with the latter species appears to bear a pro-oxidant character, inasmuch as it is associated with H_2O_2 and ferrylmyoglobin formation.

2. MATERIALS AND METHODS

2.1. *Chemicals and biochemicals*

Horse heart myoglobin was purchased from Fluka Chemical Co. (Ronkonkoma, NY, USA) and it was dialyzed against 1 mM EDTA/0.1 mM potassium phosphate buffer, pH 7.4, for 2 h prior to use. Ascorbic acid and H_2O_2 were from Sigma Chemical Co. (St. Louis, MO, USA); beef liver catalase was from Pharmacia (Uppsala, Swe-

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Abbreviations: AH_2, ascorbic acid; AH^-, ascorbate monoanion; $A^{\bullet -}$, ascorbyl radical; $Fe^{IV}=O$, oxo-ferryl complex in ferrylmyoglobin; $HX\text{-}Fe^{IV}=O$, ferrylmyoglobin; $HX\text{-}Fe^{III}$, metmyoglobin; $HX\text{-}Fe^{II}O_2$, oxymyoglobin.

den) and superoxide dismutase from Boehringer (Mannheim, Germany). Oxymyoglobin was obtained by reduction of metmyoglobin with dithionite in an oxygen-free solution as previously described [11]. Ferrylmyoglobin was formed upon incubation of equimolar amounts of metmyoglobin and H_2O_2.

2.2. *Absorption spectroscopy*

Concentrations of oxymyoglobin ($\varepsilon_{580} = 14.4$ $mM^{-1} \cdot cm^{-1}$), metmyoglobin ($\varepsilon_{630} = 2.1$ $mM^{-1} \cdot cm^{-1}$), and ferrylmyoglobin (measured at 550 and 630 nm according to the following formula: [ferrylmyoglobin] (μM) = 249 $A_{550\,nm} - 367$ $A_{630\,nm}$) were determined spectrophotometrically. Derivatization of ferrylmyoglobin to sulfmyoglobin was accomplished upon addition of Na_2S; sulfmyoglobin displayed a characteristic absorption at 621 nm ($\varepsilon = 24$ $M^{-1} \cdot cm^{-1}$) [12].

2.3. *Electron spin resonance (e.s.r.) spectroscopy*

e.s.r. spectra were recorded at 9.81 GHz on a Brucker ECS 106 spectrophotometer. Measurements were carried out with 100 kHz field modulation at room temperature. The instrument settings were: receiver gain, 2×10^6; microwave power, 10 mW; microwave frequency, 9.81 GHz; modulation amplitude, 1.527 G; time constant, 1.3 s; scan time, 5.6 min.

2.4. *Polarographic measurements*

H_2O_2 formation was measured as O_2 evolved in a reaction mixture containing different amounts of oxymyoglobin (20–80 μM) and a 5–10-fold excess of ascorbic acid in helium-purged 0.1 M potassium phosphate buffer, pH 5.5. O_2 evolution was measured with an oxygen Clark-type electron in the presence of catalase (500 $U \cdot ml^{-1}$). H_2O_2 formed was quantified taking into account that $[H_2O_2] = 0.5$ $[O_2]$.

3. RESULTS

3.1. *Ascorbic acid-mediated reduction of ferryl- and metmyoglobin*

The interaction of ascorbate with ferryl- and metmyoglobin at pH 7.4 (Fig. 1, left panel) resulted in an e.s.r. signal characterized by a doublet (1:1) with a hyperfine coupling constant of a_{H4} 1.8 G ($g = 2.00518$) and consistent with the spectrum ascribed to ascorbyl radical [13]. The ascorbyl radical e.s.r. signal intensity resulting from the interaction of ascorbate with ferrylmyoglobin was 1.45-fold higher than that originating from the interaction of the antioxidant with metmyoglobin. This higher steady-state concentration of ascorbyl radical was expected, for ascorbate reacts much faster with ferrylmyoglobin ($1.6 \pm 0.4 \times 10^6$ $M^{-1} \cdot s^{-1}$) than it does with metmyoglobin (3.3×10^2 $M^{-1} \cdot s^{-1}$). (The experimental rate constant for the former reaction was similar to that reported for the reaction of ferrylmyoglobin with the water-soluble analog of vitamin E, Trolox [14].) In the absence of hemoprotein, a low intensity ascorbyl radical signal was observed (Fig. 1C) which was accounted for by the autoxidation of ascorbate at this pH. The pK_a value for ascorbic acid is 4.25 and at pH 7.4 the concentration of the mono-anionic form (AH^-) is higher than that of the protonated form (AH_2). Ascorbic acid dissociation ($AH_2 \Leftrightarrow AH^-$) is a requisite condition for its participation in electron-transfer reactions. Indeed, at pH 5.5 ($[AH_2] > [AH^-]$), the ascorbyl radical signal originating from ascorbic acid autoxidation was abolished (Fig. 1F), whereas

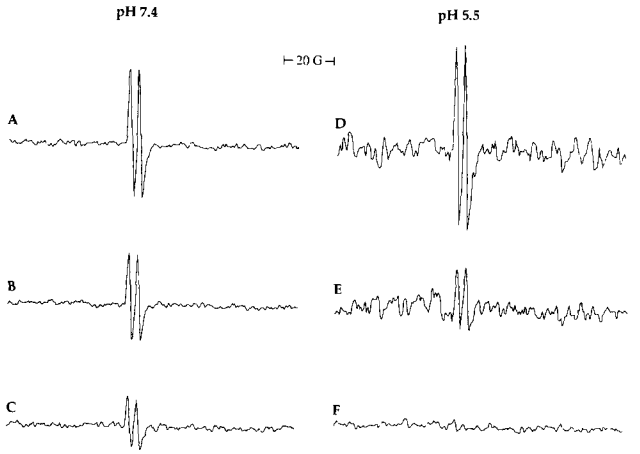


Fig. 1. Ascorbyl radical formation during the reaction of ascorbate with ferryl- or metmyoglobin. *Left panel:* the reaction mixture consisted of 1 mM ascorbic acid in 0.1 M potassium phosphate buffer, pH 7.4, supplemented with either (A) 0.2 mM ferrylmyoglobin or (B) 0.2 mM metmyoglobin; (C) no additions. *Right panel:* the same as above but at pH 5.5; (D) plus ferrylmyoglobin; (E) plus metmyoglobin; (F) no additions. Instrument settings as described in section 2. Instrument scale: left panel, 19; right panel, 17.

those arising from interaction of ascorbic acid with either ferryl- or metmyoglobin still persisted, although they were 5- and 3-fold lower, respectively, than those observed at pH 7.4 (Fig. 1, right panel). Overall, the results obtained at pH 5.5 and 7.4 indicate that the interaction of ascorbic acid with ferryl- or metmyoglobin entails a one-electron transfer process yielding ascorbyl radicals as illustrated for the case of ferrylmyoglobin in reaction **(2)**.

3.2. *Interaction of ascorbic acid with oxymyoglobin*

The interaction of ascorbate with oxymyoglobin had the following characteristic features:

First, at pH 7.4 it resulted in the formation of an ascorbyl radical e.s.r. signal, the intensity of which was similar to that observed upon reaction of ascorbate with ferrylmyoglobin and 1.4-fold higher than that with metmyoglobin (Fig. 2A). Superoxide dismutase and catalase decreased slightly (15%) the e.s.r. signal intensity (Fig. 2B,E). At pH 5.5, the ascorbyl radical signal intensity was 5.9-fold lower than at pH 7.4 (Fig. 2D), superoxide dismutase did not affect the signal intensity, and catalase decreased it as at pH 7.4 (Fig. 2C,F). The lack of effect of superoxide dismutase suggests that $O_2^{\bullet -}$ is not involved in ascorbyl radical formation at pH 5.5. The slight inhibitory effect exerted by catalase at this pH value is discussed below.

Second, the interaction between ascorbic acid and oxymyoglobin was associated with H_2O_2 formation evidenced as a catalase-mediated O_2 evolution. Horseradish peroxidase-based methods for H_2O_2 measurements could not be used in this experimental model because ascorbic acid, at the concentration used, effectively reduced compound II, thus preventing its reaction

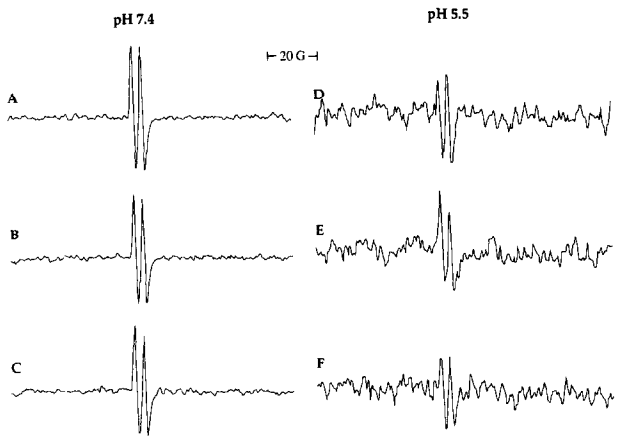


Fig. 2. Ascorbyl radical formation during the reaction of ascorbate with oxymyoglobin. *Left panel:* the reaction mixture consisted of 1 mM ascorbic acid in 0.1 M potassium phosphate buffer, pH 7.4, supplemented with 0.2 mM oxymyoglobin. (A) No additions; (B) plus 1 μM superoxide dismutase; (C) plus 500 U · ml^{-1} catalase. *Right panel:* as above at pH 5.5. Instrument settings as described in section 2. Instrument scale: left panel, 19; right panel, 17.

with the fluorescent probe. H_2O_2 formation was evaluated at pH 5.5 in order to minimize the contribution of ascorbate autoxidation to H_2O_2 steady-state. The amount of H_2O_2 formed was linearly related to oxymyoglobin concentration (Fig. 3) and dependent on the [ascorbic acid]/[oxymyoglobin] ratio: at a ratio of 5, the total yield of H_2O_2 was about 30% of the hemoprotein present, whereas at [ascorbic acid]/[oxymyoglobin] = 10, the amount of H_2O_2 formed was equimolar to the hemoprotein concentration (Fig. 3, insert).

Third, the reaction of ascorbate with oxymyoglobin was accompanied by absorption spectral changes – consisting of a small decrease in absorbance at 580 and 545 nm and a slight increase at 630 nm – ascribed to the oxymyoglobin → metmyoglobin ($Fe^{II}O_2 \rightarrow Fe^{III}$) transition (Fig. 4A). Treatment of the reaction mixture with Na_2S revealed the presence of a strong absorption at 621 nm (Fig. 4B), which is indicative of sulfmyoglobin. The occurrence of the oxoferryl complex, as in ferrylmyoglobin, is a requisite condition to observe sulfmyoglobin formation, which occurs upon sulfur nucleophilic attack onto a β-carbon atom of a pyrrole [12]. Hence, the results shown in Fig. 4B reveal the presence of an oxoferryl intermediate during the interaction of oxymyoglobin with ascorbate. These findings also explain the slight inhibitory effect of catalase on e.s.r. signal intensity corresponding to ascorbyl radical, indicating the contribution of ferrylmyoglobin – formed upon interaction of metmyoglobin with H_2O_2 – to ascorbyl radical formation.

The experimental evidence presented here, entailing the formation of ascorbyl radical and H_2O_2 as well as that of an oxoferryl complex intermediate, is consistent with a mechanism encompassed by the following sequence. (a) A concerted electron transfer to O_2 in oxymyoglobin yielding ascorbyl radical, metmyoglobin, and H_2O_2. The formation of ascorbyl radical and metmyoglobin (implying one-electron oxidations), indicates that the two electrons required for the reduction of O_2 to H_2O_2 are contributed by ascorbate and the heme iron. This view further suggests that oxymyoglobin occurs partly as superoxoferrimyoglobin (HX-$Fe^{III}O_2^{\bullet -}$) [15] in order to facilitate the initial oxidation of ascorbate (reaction 3). (b) Rapid oxidation of metmyoglobin by H_2O_2 yielding ferrylmyoglobin (reaction **(1)**

$$[\text{HX-Fe}^{\text{II}}\text{O}_2 \Leftrightarrow \text{HX-Fe}^{\text{III}}\text{O}_2^{\bullet -}] + \text{AH}^- + \text{H}^+ \rightarrow$$

$$\text{HX-Fe}^{\text{III}} + \text{H}_2\text{O}_2 + \text{A}^{\bullet -} \qquad \textbf{(3)}$$

above). (c) Reduction of the oxoferryl derivative to metmyoglobin as indicated in reaction **(2)** ($k_2 = 1.6 \pm 0.4 \times 10^6$ M^{-1} · s^{-1}) and the experimental observations in Fig. 1 and previous reports [8–10]. Steps (b) and (c) above explain the lack of direct absorption spectral evidence for ferrylmyoglobin (i.e., only the oxymyoglobin → metmyoglobin transition in Fig. 4A is observed) and the necessity to displace this species from the equilibrium upon its derivatization to sulfmyoglobin (Fig. 4B).

4. DISCUSSION

In this study, we provided evidence that ascorbyl radical is indeed generated during the reduction of ferrylmyoglobin by ascorbate as previously suggested by monitoring the ferrylmyoglobin → metmyoglobin conversion. The antioxidant feature inherent in this reaction is suggested by the removal of ferrylmyoglobin (a strong oxidant with a reduction potential of about +1.4 V) [16] and transfer of the radical character to ascorbate; the ascorbyl radical is a weak oxidant (+0.28 V)

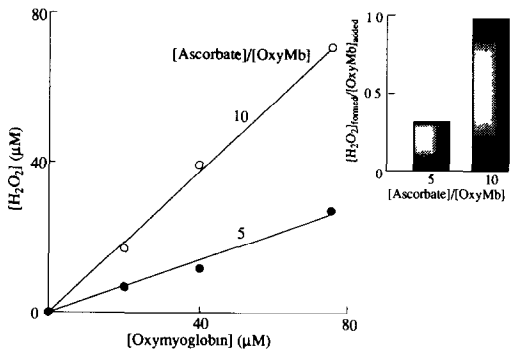


Fig. 3. H_2O_2 formation during the reaction of ascorbate and oxymyoglobin. Assay conditions: different amounts of oxymyoglobin (10–80 μM) in helium-purged 0.1 M potassium phosphate buffer, pH 5.5, were supplemented with ascorbic acid (to reach a [ascorbic acid]/[myoglobin] ratio of 5 or 10. Insert: $[H_2O_2]_{formed}/[\text{oxymyoglobin}]_{added}$ ratio vs. [ascorbic acid]/[oxymyoglobin]. Abbreviation: oxyMb, oxymyoglobin.

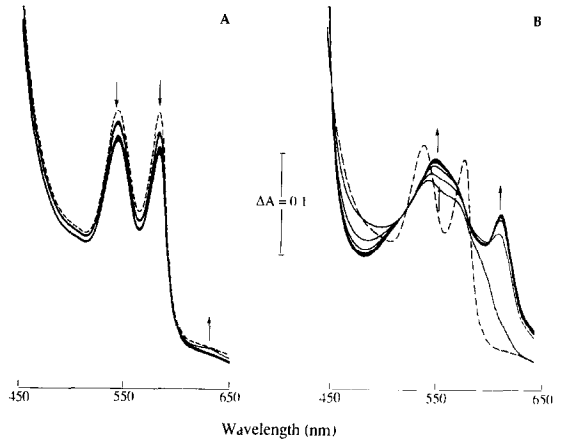
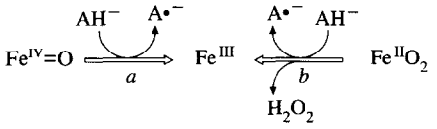


Fig. 4. Visible absorption spectral changes during the reaction of oxymyoglobin with ascorbate. (A) Assay conditions: 50 μM oxymyoglobin in 0.1 mM potassium phosphate buffer, pH 7.4, was supplemented with 2 mM ascorbic acid to initiate the reaction. (---) oxymyoglobin; (—) oxymyoglobin plus ascorbic acid. Scans were taken at 1 min intervals. (B) Assay conditions: oxymyoglobin was incubated with ascorbate for 10 min (as in (A)) (---). After this period, 1 mM Na_2S to derivatize ferrylmyoglobin to sulfmyoglobin (—). The upward and downward arrows indicate increase and decrease in absorbance, respectively. Scan intervals: 1 min.

[17], which can readily decay by disproportionation to nonradical products [18]. This, along with the proposed role for ferrylmyoglobin in coronary heart diseases [3–5] and the reported protective effect of ascorbate in induced ischemic arrest associated with cardiopulmonary bypass [16], suggests a potential antioxidant function for ascorbate against oxidative damage during certain cardiomyopathies.

The interaction of ascorbate with oxymyoglobin, encompassing the sequence of reactions **(3)-(1)-(2)** above, is reminiscent of the reaction of oxyhemoglobin with acetylphenylhydrazine [20] and with NO_2^- [21]; in the former case, a methemoglobin–H_2O_2 intermediate complex was proposed, although it was not identified as the oxoferryl complex of ferrylhemoglobin, whereas in the latter case, ferrylmyoglobin was shown to be an intermediate. In this study, the identification of potent oxidant, such as ferrylmyoglobin, ensuing from the interaction of ascorbate with oxymyoglobin, was accomplished



(4)

by its derivatization to sulfmyoglobin (Fig. 4B). The occurrence of this oxoferryl complex intermediate suggests the prooxidant character of this redox transition.

The interactions of ascorbic acid with different heme iron redox states of myoglobin reveal antioxidant and prooxidant features: the former is substantiated by the *reduction* of ferrylmyoglobin by ascorbate (reaction **(4)***a*), whereas the latter by the *oxidation* of oxymyoglobin by ascorbate, in the course of which a ferrylmyoglobin intermediate is formed (reaction **(4)***b*). Evaluation of the significance of the latter aspect requires consideration of absolute rate constants and steady-state concentrations of reactive intermediates. Although it could be argued that the oxoferryl intermediate is readily reduced back to metmyoglobin by ascorbate (as in reaction **(2)**), thus preventing its oxidative interaction with bioconstituents, it is also possible that in appropriate cellular settings other electron donors would compete efficiently with ascorbate. The radical species formed during this interaction may not be a weak oxidant as the ascorbyl radical and it could propagate oxidative reactions.

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