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Studies of the Reactions of Iron(II) Ascorbate Mixtures with Molecular Oxygen in Solution

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Abstract

Mössbauer and electronic absorbance spectrossopic studies on the reactions of iron(II):ascorbic acid with molecular oxygen in aqueous and methanolic solutions are reported. Both spectroscopic techniques show that in the starting mixtures there are no iron(II):ascorbate complexes. On mixing the iron(II)/ascorbate solution with solutions containing molecular oxygen at pH 6–7 high spin iron(III) is observed in the Mössbauer spectrum. Coloured intermediates, the lifetimes of which are solvent dependent, are seen by stopped-flow spectrophotometry. We assign these coloured intermediates as iron(III) ascorbate complexes. The stoichiometry of the initial reaction between iron(II) and oxygen is shown to be 2Fe(II):O_2 by stopped-flow methods. A scheme for the overall reactions is discussed.

Introduction

A number of studies have been undertaken to examine the reactions between ferric iron and ascorbic acid [1–4]. In a rapid kinetic investigation [5] of the reactions of iron(III) with ascorbate we have reported the transient appearance of a number of coloured intermediates the nature of which depend upon solution conditions e.g. pH and whether aqueous or methanolic. These studies, together with Mössbauer spectroscopy have led us to suggest a mechanism in which iron(III) becomes reduced to iron(II) under acidic conditions via an iron(II)–ascorbate radical intermediate. This latter complex is blue ($\lambda_{max} = 650$ nm) due to ligand to metal charge transfer bands. The presence of coloured intermediates in the reduction of iron(III) by ascorbate is reminiscent of the analogous reactions with glutathione [6]. In that case, however, it is an iron(II)–sulphur radical complex which is thought to be responsible for the colour. Iron(III) reduction by catechols also exhibits similar properties [7].

In this report we have turned our attention to the product of the reaction of iron(III) ascorbate mixtures, (*i.e.* iron(II) largely coordinated to solvent [5], in the presence of excess ascorbate) with molecular oxygen. The analogous reaction with the glutathione/iron(II) system shows that the iron is rapidly oxidized by oxygen. The presence of transient coloured species on the pathway has allowed us to measure the iron to oxygen stoichiometry as two iron atoms to one oxygen atom [8]. This result suggests that hydrogen peroxide is formed as a product. It is of interest to enquire if a similar mechanism and stoichiometry pertains to the reaction of iron(II) with oxygen in the presence of ascorbic acid.

Our experiments with the ascorbate/iron(II) system do in fact show coloured intermediates on reaction with oxygen but of somewhat different nature to those formed when glutathione is the reductant [8]. The stoichiometry, however, remains at two irons to one oxygen. The mechanism, as it appears to involve the production of H_2O_2, may have bearing on the inflammatory effects of 'free' iron *in vivo* [9, 10]. It has also been reported that the dehydro ascorbate/ascorbate ratio is elevated in the synovial fluid and serum from arthritic patients. Parts of this increase may be due to the presence of 'free' iron catalyzing ascorbate oxidation with consequent peroxide formation and inflammation.

Experimental

(I) Stopped-flow Kinetics

Experiments were performed using a Durrum stopped-flow apparatus with a 2 cm path length cell and dead-time of 3 to 4 ms.

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(a) Methanolic solutions

Iron(II)/ascorbate mixtures were prepared in 80% methanol under nitrogen by mixing 4 X 10^{-4} M $FeCl_2 \cdot 4H_2O$ with an equal volume of 4 X 10^{-3} M sodium ascorbate. The resulting solutions were colourless and the apparent pH 6.7. These solutions were transferred under positive pressure of nitrogen to syringes which could be fitted to the stopped-flow apparatus. The other storage syringe of the apparatus contained 80% methanol at pH 6.7 and oxygen at known concentrations.

Solutions of known oxygen concentrations were prepared by diluting either air saturated or oxygen saturated 80% methanol. The oxygen content of the air and oxygen saturated 80% methanol was determined polarographically with an oxygen electrode using oxygen saturated water at 20 °C (260 μM oxygen) as a reference.

In other experiments acidic iron(II)/ascorbate methanolic solutions were prepared under nitrogen then mixed in the stopped-flow apparatus with oxygen containing basic methanolic solutions. The resultant pH of the outflow was measured at pH 6.9.

(b) Aqueous solutions

Iron(II) chloride/ascorbic acid mixtures were prepared under nitrogen and adjusted to pH 4.1 with 1 M NaOH. The procedure was continued as for the methanolic solutions.

(II) Decay of the Intermediate

Iron(II)/ascorbate solutions, with an iron to ascorbate ratio of 1:10 were prepared under nitrogen in 80% methanol, $[FeCl_2 \cdot 4H_2O] = 2 \times 10^{-4}$ M, pH 6.7. The solutions were equilibrated with air until the purple colour was fully developed then the decay of the colour was monitored with time using a Perkin-Elmer 575 UV–Vis spectrophotometer.

(III) Mössbauer Spectroscopy

Iron(II)/ascorbate solutions were prepared under nitrogen in 80% methanol pH 6.5, by mixing 0.1 M $FeCl_2 \cdot 4H_2O$ with a 10-fold excess of sodium ascorbate. The colourless solutions were frozen and the Mössbauer spectra recorded at 80 K. The material was then thawed, exposed to air, shaken and the resulting purple solutions frozen and their spectra recorded.

The Mössbauer spectra were obtained on a spectrometer described previously [6]. The spectrometer was calibrated with a 25 μm thick iron reference absorber. All isomer shifts are referred to this as zero shift.

Results and Discussion

In a similar manner to the thiol/iron system [8], around neutral pH values, iron(II) solutions containing ascorbate gave an intense purple colour when shaken with molecular oxygen. Under strictly anaerobic conditions, however, neither aqueous nor methanolic solutions of iron(II)/ascorbate mixtures showed any absorption in the visible region suggesting that the iron(II) was not coordinated to ascorbate but to solvent, this is in agreement with Mössbauer data presented below. It is also consistent with the reported very low stability constants for iron(II) ascorbate complexes ($K \sim 10^{-3}$ M^{-1}) [12, 13]. Thus an iron(II) ascorbate complex is not a precondition for the reaction with oxygen (*cf.* the thiol system [8]).

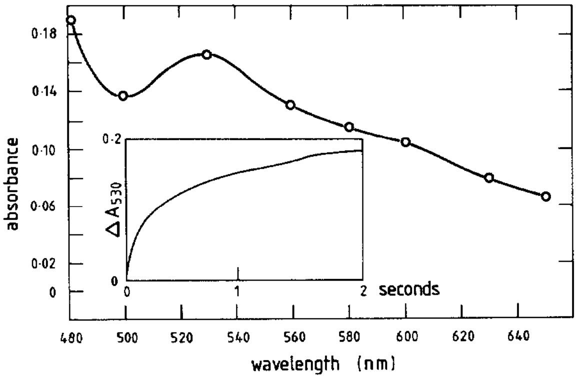

Fig. 1. Electronic absorption spectrum of the intermediate formed in water by reacting Fe^{2+}/ascorbate with oxygen. The spectrum was calculated from the amplitude of progress curves as shown in the insert, the conditions for which were $[Fe^{2+}] = 2 \times 10^{-4}$ M; [ascorbate] = 2×10^{-3} M and $[O_2]$ = 120 μM after mixing. The temperature was 25 °C, the pH = 4.1 and the monitoring wavelength for the insert 530 nm.

Stopped-flow Kinetics

(a) Formation of an intermediate complex in the oxygen reaction with iron(II)/ascorbate in aqueous solution

In order to examine the properties of any intermediate and obtain rate data to elucidate mechanism, rapid-mixing experiments were performed in a stopped-flow apparatus. On mixing oxygen containing solutions with iron(II)/ascorbate at pH 4.1 a rapid increase in absorbance in the visible spectrum was observed (see insert Fig. 1). This indicated the rapid formation of a reddish complex which then decayed in a slower process to a colourless product.

By performing experiments at a variety of wavelengths, it was possible to build up the absorption spectrum of the coloured complex. Figure 1 shows the broad absorbance band of the intermediate with a maximum at 530 nm. Assuming all the iron in solution is involved in the formation of the red complex, it is possible to calculate its extinction coefficient as 500 cm^{-1} M^{-1}.

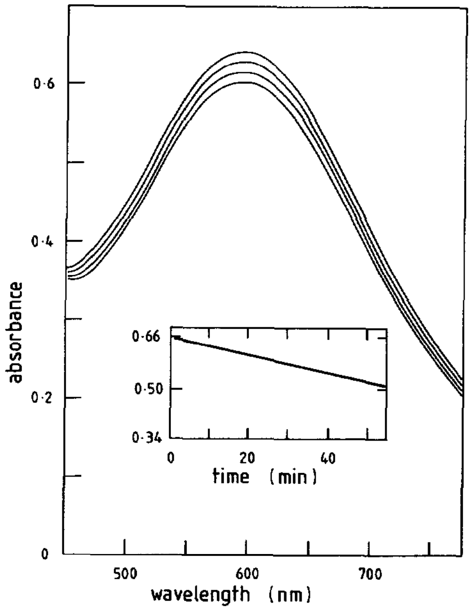

Fig. 2. Static spectrum of the complex formed on mixing Fe^{2+}/ascorbate mixtures with oxygen at pH 6.7. [Fe^{2+}] was 2×10^{-4} M in the presence of a 10-fold excess ascorbate and in 80% methanol equilibrated with air. Spectra (1), (2), (3) and (4) were recorded at 10 min intervals. The insert shows the decay of the absorbance at 595 nm as a function of time.

Due to the instability of the coloured intermediate [5], because of ligand exchange, further kinetic studies were not undertaken at this time in aqueous solution (the red colour decays after 2 seconds). In order to investigate this intermediate further, therefore, studies were performed in methanolic solutions since the latter stabilizes the intermediates [5].

(b) Formation of the purple intermediate in 80% methanol

Comparable experiments to those reported above were carried out in 80% methanol. The formation of a purple species, which was much longer lived than that in water, resulted, allowing a static spectrum (Fig. 2) to be recorded. Kinetic experiments on the formation of this species were performed in two ways:

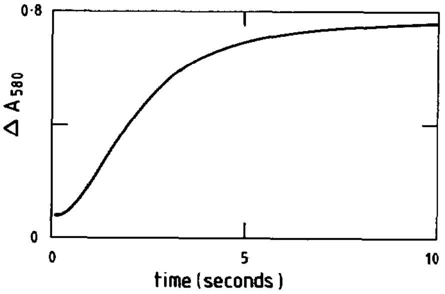

Fig. 3. A progress curve showing the reaction of Fe^{2+}/ascorbate with oxygen in 80% methanol. λ = 580 nm, [Fe^{2+}] = 2×10^{-4} M, [ascorbate] = 2×10^{-3} M and [O_2] 0.77×10^{-4} M, after mixing.

(1) Both iron(II)/ascorbate solution and the oxygen containing solution were adjusted to pH 6.7 before mixing. The anaerobic solution when first prepared was colourless but started developing slight colouration on standing (because of its high sensitivity towards oxygen which slowly leaked into the stopped-flow syringe). Therefore, the data obtained from this experiment are not suitable for the determination of the O_2/Fe stoichiometry through the amplitude of the absorbance changes; kinetic data can, however, be obtained. The time courses (see Fig. 3 for example) are similar to those we have observed [5] for the reaction or iron(III) with ascorbate in 80% methanol. The spectrum of the final product of the reaction between the iron(II)/ascorbate system and oxygen is also identical, within our errors, to that of the reaction between iron(III) and ascorbate.

(2) Anaerobic iron(II)/ascorbic acid solutions at apparent pH ~2.5 in 80% methanol were mixed in the stopped-flow apparatus with basic methanol (80%) containing oxygen. The pH after mixing was in the range pH 6–7.

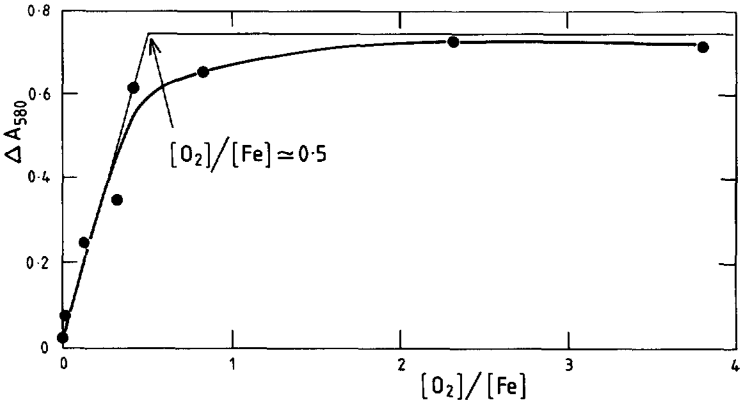

Fig. 4. Change in Δ absorbance at λ_{max} (580 nm) of the purple complex at pH 6.9 as a function of [oxygen]:[iron] ratio. [Fe^{2+}] = 2×10^{-4} M after mixing and in the presence of a 10-fold excess ascorbate.

TABLE I. ^{57}Fe Mössbauer Parameters of Frozen Solutions at 78 K

	∂ (mm s^{-1})	Δ (mm s^{-1})	Γ (mm s^{-1})	*A* (%)
(1) Fe(II)/asc anaerobic (80% methanol) pH 6.5	1.39(2)	3.07(4)	0.27(3)	100(10)
(2) Sample (1) + O_2	1.39(2)	3.07(4)	0.27(3)	89(11)
	0.53(4)	0.45(5)	0.09(5)	11(7)
(3) Fe(II)/asc + O_2 (water) pH 6.5	1.34(2)	2.94(4)	0.28(3)	77(10)
	0.36(4)	0.29(5)	0.17(5)	23(9)

The advantage of this experimental procedure is that it allows accurate determination of the stoichiometry between O_2 and Fe for complex formation. This is because the starting materials were both colourless *i.e.* no net oxidation of the iron(II) occurs at low pH as the iron(III) is rapidly reduced by ascorbate at pH 3 [5]. The stoichiometry of O_2/Fe was calculated from monitoring the maximum change in absorbance at 580 nm as a function of oxygen concentration. Figure 4 presents the results of such an experiment. The absorbance increases with oxygen concentration up to a plateau after which no further increase is observed. The maximum production of the purple product is reached at an O_2/Fe ratio of close to 1:2. The valence of the iron in the purple complex is discussed below.

Mössbauer Spectroscopy

The Mössbauer parameters of anaerobic solutions of iron(II)/ascorbate (Table I) showed the presence of high spin iron(II) as expected. The data are similar to others we have reported previously [5, 6, 8] and indicate that iron(II) is in a chloride and solvent molecule ligand environment.

In both methanolic and aqueous solutions along with the formation of the purple intermediate, we have now observed a fraction of the iron which, from the parameters, is high spin iron(III) ascorbate complex (see ref. 1). The quantitation of this signal is reported in Table I. This is likely to be a rough estimate of the concentration of iron(III) at 78 K as the iron(II) and iron(III) may not have equivalent recoil free fractions in these frozen solutions. Also as the iron(III) concentration was 0.1 gram ion/litre it is difficult in a short period of shaking with air to introduce sufficient oxygen into the solution to oxidize the majority of the iron. It is clear from the three Mössbauer spectra that the introduction of oxygen is responsible for the formation of the iron(III) site.

Conclusions

By comparison with the results of investigation on iron(III)/ascorbate reactions at high pH ~6–7 [5] we conclude that the purple intermediate observed in the reaction of iron(II)/ascorbate mixtures with oxygen is in fact an iron(III)–ascorbate complex. The absorbance changes we observed in the stopped-flow apparatus correspond to the build up of this complex [5]. The Mössbauer spectra and, previously reported, very low stability constants [12, 13] indicate that iron(II)–ascorbate complexes are not present in the initial reactants. This suggests that iron(II) is first oxidized by molecular oxygen and the resulting iron(III) then coordinates to ascorbate to produce the purple species.

Kinetic titrations of iron(II)/ascorbate by molecular oxygen in methanolic solutions (where the purple species is relatively stable), show that complex production reaches a maximum at an iron to oxygen ratio of 2:1. As each iron(II) donates a single electron we interpret this result to suggest that, even if superoxide anions participate in the reaction, the final product of the oxygen reaction is hydrogen peroxide. This mechanism is similar to that proposed in the oxidation of iron(II)/glutathione systems by molecular oxygen [8] with the exception that there the iron(II) is complexed to glutathione before the reaction with oxygen is initiated.

The reaction between oxygen and iron(II)/ascorbate can be represented as follows:

$$2Fe^{2+} + O_2 \longrightarrow 2Fe^{3+} + O^{2-} \tag{1}$$

$$Fe^{3+} + HAsc^{-} \longrightarrow [Fe(III)AscH]^{2+} \text{ purple} \tag{2}$$

$$2H^{+} + O_2{}^{2-} \longrightarrow H_2O_2 \tag{3}$$

Equation 1 probably proceeds via a two step reaction, possibly involving the superoxide anion, which then disproportionates. This equation however preserves the stoichiometry observed in the experiments shown in Fig. 4. We suspect reactions of the type

$$2Fe^{2+} + H_2O_2 \longrightarrow 2Fe^{3+} + 2OH^{-}$$

must proceed slowly (compared to our kinetic measurements) under these conditions as otherwise the stoichiometry given in eqn. (1) would not be observed.

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