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Reduction of oxidized cytochrome *c* by ascorbate ion

Nicola H. Williams and John K. Yandell

Chemistry Department, Monash University, Clayton, Victoria, 3168 (Australia)

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The kinetics and mechanism of the reduction of oxidized cytochrome *c* by ascorbate has been investigated in potassium nitrate, potassium 4-morpholineethanesulfonate (KMes), potassium sulfate and potassium ascorbate media. The results are consistent with simple second order electron transfer from ascorbate dianion to cytochrome *c* and do not support electron transfer from an ascorbate dianion bound to the protein of the cytochrome as recently proposed by Myer and Kumar. A rate constant of $8 \cdot 10^5$ $M^{-1} \cdot s^{-1}$ (25°C, ionic strength, 0.1) was found for the electron-transfer step. This rate constant is essentially independent of the specific ions used in controlling ionic strength.

Oxidation of ascorbic acid (H_2A) and ascorbate monoanion (HA^-) by one electron oxidants with low redox potentials has been shown [1,2] to proceed by one electron transfer from ascorbate dianion (A^{2-}). Reduction of oxidized cytochrome *c* by low concentrations of ascorbate ion (less than 10 mM) has been found [1,3] to fit the general pattern of ascorbate reactions, and it has therefore been proposed that the mechanism of the reaction can be represented by

$$HA^- \rightleftarrows H^+ + A^{2-} \quad (1)$$

$$\text{cytochrome } c(\text{III}) + A^{2-} \rightarrow \text{cytochrome } c(\text{II}) + \dot{A}^- \quad (2)$$

$$\dot{A}^- + \text{cytochrome } c(\text{III}) \rightarrow A + \text{cytochrome } c(\text{II}) \quad (3)$$

where reaction (1), $pK_a = 11.45$, is a rapid preequilibrium to the rate determining electron transfer reaction (2) ($\dot{A}^-$ and A are ascorbate radical ion and dehydroascorbic acid, respectively). In addition, the rate constant for electron transfer is close to that expected [1] for simple outer-sphere electron transfer, presumably through the heme edge.

Recently, however, Myer and Kumar [4] concluded that this simple mechanism is not applicable to cytochrome *c* reduction, and instead proposed that electron transfer occurs from ascorbate dianion bound to the protein at a site 1.4 to 2.0 nm from the heme. Furthermore, they claim that the electrons tunnel, with low activation energy, between the bound ascorbate and the heme. These conclusions were based on deviations from linearity of plots of observed rate constant vs. ascorbate concentration. Deviations of less than 15% were found in the range 50–80 mM ascorbate. Meyer and Kumar interpreted this curvature in terms of a preassociation of ascorbate dianion with the protein of cytochrome *c*. The work described in this paper does not support this interpretation, but is instead consistent with the simple mechanism described by reactions (1), (2) and (3).

We have extended the investigation of the ascorbate-cytochrome *c* reaction to much higher ascorbate concentrations than employed by Meyer and Kumar, and we find no evidence of a non-lin-

Abbreviations: Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; (K)Mes, (potassium) 4-morpholineethanesulfonic acid.

ear dependence on ascorbate concentration (Fig. 1). This suggests that the non-linear behaviour reported by Myer and Kumar is an artifact of their methodology. They employed a medium containing 0.25 M sodium sulphate/0.05 M phosphate (at pH 7.3) and varied the ascorbate concentration from 10 to 80 mM. In our studies 1 : 1 electrolytes (KNO_3 and K(Mes)) were used to maintain a constant concentration of ions. Thus the only change in the medium as the potassium ascorbate concentration was increased was the replacement of one monoanion (NO_3^- or Mes^-) by another (HA^-).Under these conditions linear behaviour is observed up to 0.25 M ascorbate (Fig. 1), at which concentration deviations of 50% or more would be expected by extrapolation of Myer and Kumar's data.

We have also examined the effect of the ionic medium on the rate of the ascorbate-cytochrome *c* reaction (Table I) in an attempt to explain Myer and Kumar's observations. We, as well as others [5], found the reaction to be sensitive to changes in the ionic medium. With 1 : 1 electrolytes a decrease

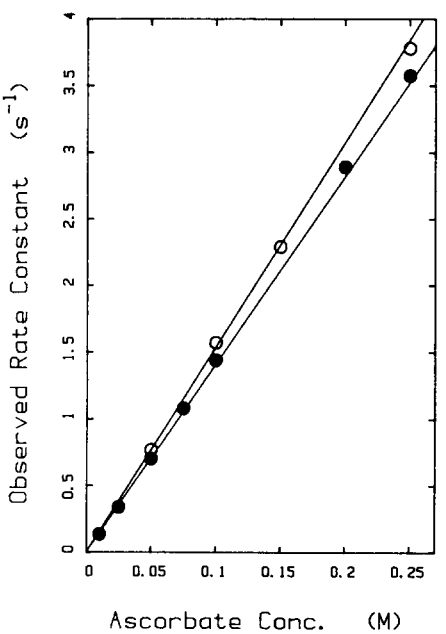

Fig. 1. Rate constants for reduction of cytochrome *c*(III) by ascorbate ions. Conditions: pH 7.3, 25°C; ●, K^+ concn., 0.5 M; nitrate concn. + ascorbate ion concn., 0.5 M; ○, K^+ concn., 0.5 M, Mes^- concn. + ascorbate ion concn., 0.5 M; cytochrome *c*, approx. $5 \cdot 10^{-6}$ M.

TABLE I

RATE CONSTANTS FOR REDUCTION OF CYTOCHROME *c*(III) WITH ASCORBATE IONS IN DIFFERENT MEDIA

Conditions: 25°C, pH 7.3; all solutions were made up to the appropriate ionic strength with the salt given in the table; rate constants were determined at low ascorbate concentrations (not more than 10 mM) except in K(HA) medium; 0.01 M Hepes buffer was used except in K(Mes) medium.

Ionic strength	Rate constants ($M^{-1} \cdot s^{-1}$)			
	KNO_3	K(Mes) [a]	K(HA) [b]	K_2SO_4
0.1	51	50	42	45
0.5	14	15	15	25
0.75				22 (20 [c])

[a] Potassium salt of morpholinoethanesulphonic acid.
[b] Potassium (ascorbate monoanion).
[c] From Ref. 4.

of a factor of 3–4 is observed in the second-order rate constant on increasing the ionic strength from 0.1 to 0.5 M. Most importantly, this difference is observed in KNO_3 and K(Mes) solutions, and in solution in which only potassium ascorbate is present. This strongly implies that the decrease is a consequence of a general effect of the ionic medium, and specifically that the decrease in the potassium ascorbate solutions is not the result of saturation of an ascorbate binding site but of this general ion inhibition. The small decrease in rate constant with increasing ascorbate concentration in Myer and Kumar's experiments can also be ascribed to this general effect.

In K_2SO_4 medium, non-linear behaviour was observed (Fig. 2) even when the total charge of the medium was kept constant, though the curvature is much less than in Myer and Kumar's medium which had a fixed sulfate concentration. Apparently, the non-linearity arises because the rate constants observed in 0.25 M K_2SO_4 solutions are greater than those in high concentrations of 1 : 1 electrolytes, so that increasing the potassium ascorbate concentration results in a decrease in the rate constant (Table I). These observations reinforce our conclusion that the non-linearity in K_2SO_4 solutions is a consequence of a general ion effect and not of binding of ascorbate to specific sites in the protein of cytochrome *c*.

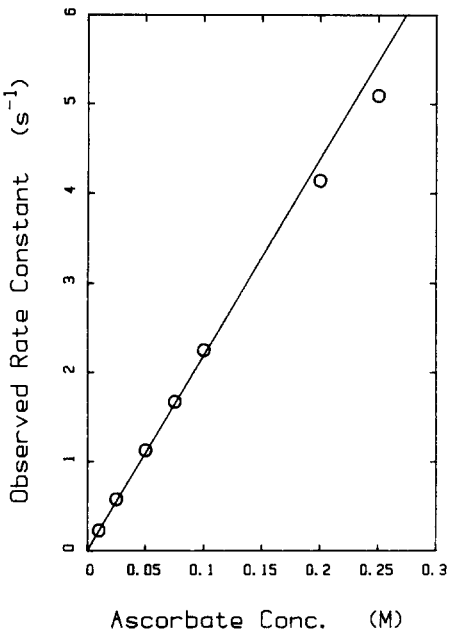

Fig. 2. Rate constants for reduction of cytochrome *c*(III) by ascorbate ions; sulfate medium. Conditions: pH 7.3, 25°C, K^+ concn., 0.5 M, 2×sulfate concn. + ascorbate ion concn. 0.5 M; cytochrome *c* approx. $5 \cdot 10^{-6}$ M.

We conclude, therefore, that the simple mechanism represented by reactions (1)–(3) with electron transfer through the solvent exposed heme edge, is still the most plausible mechanism for the reduction of cytochrome *c*(III) by ascorbate.

Two further points can be made in support of this proposal. First, the simple mechanism predicts that the rate of reaction should be proportional to the reciprocal of the hydrogen ion concentration. This has been demonstrated by Al-Ayash and Wilson [3] for a number of cytochromes and is supported by our data (Fig. 2). In contrast, Myer and Kumar's mechanism leads to an unexplained pH dependence for electron transfer. Second, the rate constant for the electron-transfer step (reaction (2)) is close to that expected for simple electron transfer. The rate constant derived from our data is $(8 \pm 1) \cdot 10^5$ $M^{-1} \cdot s^{-1}$ (25°C, ionic strength, 0.1) compared with the value of $(1\text{–}4) \cdot 10^6$ $M^{-1} \cdot s^{-1}$ derived from the Marcus cross relation [1,2]. Considering the assumptions made in this calculation [6], particularly the use of the Debye-Hückel equation, it is unlikely that the difference is significant.

Finally, we would like to comment on the possibility of long distance electron transfer (or tunneling) through the protein in the manner proposed by Myer and Kumar. Marcus and Sutin [6] have recently reviewed the general background to electron 'tunneling' over long distances, and have specifically analysed the systems of Isied and coworkers [7] and Nocera et al. [8] involving electron transfer to the heme from an electron donor bound to the protein of cytochrome *c*. The expression $\exp\{-\beta(r - r_0)\}$ was used to estimate the tunneling probability over distance *r*. (r_0 is the distance at which the probability of electron transfer is 1, and β is a constant estimated to be 12 nm^{-1}). This approach suggests that the rate of electron transfer from a binding site 1.4 nm from the heme (as proposed by Myer and Kumar) would be from 10^{-5}–10^{-8} slower than that from a site at or near the heme edge. An association constant of 10^5–10^8 for binding at the 1.4 nm site is, therefore, neces-

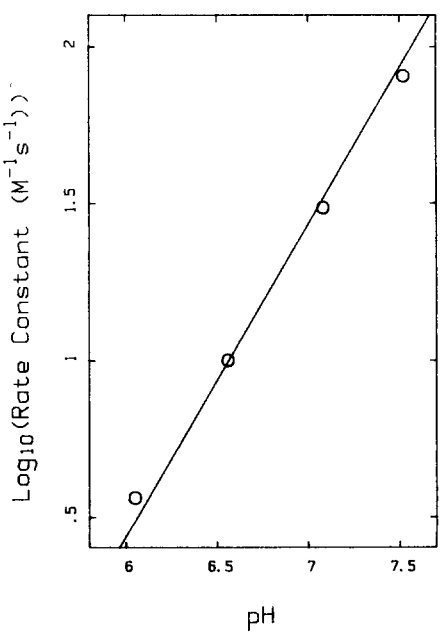

Fig. 3. Second order rate constants for reduction of cytochrome *c*(III) by ascorbate ions. Line drawn has a slope of 1.0. Conditions: 25°C, ionic strength 0.1 M (KNO_3). Mes and Hepes buffers (0.01 M); ascorbate ion concentration 0.05 M; cytochrome *c* concn. approx. $5 \cdot 10^{-6}$ M.

sary to compensate for the lower probability of electron transfer. Thus electron transfer from a remote site on the protein is feasible provided a binding site of sufficiently high affinity is present. This remains to be established in the ascorbate-cytochrome system.

Materials: horse heart cytochrome *c* (oxidized, Type VI, Sigma); L(+) Ascorbic acid; 'Analar' potassium sulphate and potassium nitrate (May and Baker); Hepes and Mes (Sigma); and 'Convol' potassium hydroxide (B.D.H.) were used without further purification.

Solutions: solutions for the ascorbate dependence studies (Fig. 1) on the stopped flow were prepared in the following way. A stock solution of ascorbate (0.5 M) was prepared from ascorbic acid and KOH and the pH adjusted to 7.3. Stock solutions of KNO_3, K_2SO_4 and K(Mes) (K^+ concn., 0.5 M) with 0.01 M Hepes buffer were also adjusted to pH 7.3. These solutions were used to dilute the ascorbate stock to the required concentration and also for the cytochrome *c* solutions (approx. 4 μM). All solutions were 10 μM in EDTA. Other solutions were prepared in a similar manner.

Kinetics: all runs were performed with a pseudo-first-order excess of ascorbic acid, at a monitoring wavelength of 550 nm, on a stopped-flow spectrophotometer. Rate constants were obtained from computer plots of log(absorbance$_\infty$ − absorbance$_t$) against time (t), which were linear for at least two half lives.

Apparatus: pH meter, Metrohm Herisau Präzision EF10; stopped-flow spectrophotometer, adapted from a design provided by P.A. Tregloan, University of Melbourne.

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