Biochem. J. (1979) 177, 641–648
Printed in Great Britain

The Mechanism of Reduction of Single-Site Redox Proteins by Ascorbic Acid

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(*Received* 24 *July* 1978)

The reduction of single-site haem and copper redox proteins by ascorbic acid was studied as a function of pH. Evidence is presented that indicates that the double-deprotonated ascorbate anion, ascorbate^{2-}, is the reducing agent, and the pH-independent second-order rate constants for reduction by this species are given. Investigation of the temperature dependences of these rate constants have yielded the values of the activation parameters (ΔH^* and ΔS^*) for reduction. These values, together with ligand-replacement studies, suggest that ascorbate^{2-} acts as an outer-sphere reductant for these proteins. Reasons to account for the apparent inability of ascorbic acid to reduce the alkaline conformer of mammalian ferricytochrome *c* are suggested.

Ascorbic acid (vitamin C) has been extensively used as a reducing agent in the study of redox proteins. The advantages of ascorbic acid lie in the fact that it has a sufficiently low redox potential (Sober, 1970) to reduce such proteins as *c*-type cytochromes (from both eukaryotes and prokaryotes) and the blue copper proteins (e.g. azurin), and also that, in the absence of heavy-metal ions and at neutral pH, it is only slightly autoxidizable. In addition it may be used as an electron donor for phosphorylating preparations without the loss of phosphorylation (Minnaert, 1961).

The physiological and biochemical roles of ascorbic acid as a vitamin C are well established, yet the chemistry and the mechanism by which ascorbate reduces haem proteins, and cytochromes in particular is not completely understood. Several workers have reported that the rate of reduction of haem proteins by ascorbic acid or its sodium salt is pH-dependent, e.g. horse heart cytochrome *c* (Greenwood & Palmer, 1965) and mammalian cardiac cytochrome c_1 (Kaminsky *et al.*, 1975). This has been interpreted by Kaminsky *et al.* (1975) to be a reflection of some pH-dependent change in the protein, i.e. the enhanced rate of reduction of cytochrome c_1 by ascorbate is considered to be a consequence of the increased accessibility of the haem ion to ascorbate. Kaminsky *et al.* (1975) have related this pH-dependent increase in rate of reduction of cytochrome c_1 to conformational or ligand-exchange processes in the protein by reference to the bleaching of the 695nm absorption band on increasing pH.

An alternative proposal is that the properties of ascorbic acid as a reducing agent are themselves pH-dependent. In the present paper we present a model that accounts for the pH-dependent properties of ascorbic acid acting as a reducing agent, the central feature of which is that ascorbic acid exists in two forms, in pH-dependent equilibrium, and that only the doubly deprotonated form can act as a single-electron reducing agent, i.e.:

$$\text{H–ascorbate}^- \overset{K}{\rightleftharpoons} \text{ascorbate}^{2-} + \text{H}^+ \quad (1)$$

$$\text{Ascorbate}^{2-} + \text{oxidized redox protein} \xrightarrow{k_1} \text{reduced redox protein} + \text{dehydroascorbate} \quad (2)$$

The ionization of eqn. (1) is known to occur in ascorbic acid with a pK of 11.57 at 18°C (Birch & Harris, 1933). In the present paper we report experiments that support this model. Also, in an attempt to explore the mechanism by which ascorbate^{2-} reduces redox proteins we have performed experiments to distinguish between inner- and outer-sphere mechanisms. These experiments fall into two categories: (*a*) an examination of the temperature-dependence of the intrinsic rate constant for the reduction (k_1); (*b*) a comparison of the rate of reduction of cytochrome *c* by ascorbate^{2-} with the rate of 'crevice' opening in this protein.

Materials and Methods

Horse heart cytochrome *c* [type III; Sigma (London) Chemical Co., Kingston-upon-Thames, Surrey, U.K.] was used without further purification. *Pseudomonas aeruginosa* cytochrome c_{551} and azurin were gifts from Dr. C. Greenwood (University of East Anglia, Norwich, U.K.). *Coryphaena hippurus* (dolphin fish) cytochrome *c* was a gift from Dr. J. Bannister (Royal University of Malta, Malta). *Dictyostelium discoideum* cytochrome *c* was prepared as described by Al-Ayash & Wilson (1977). Guanidinated cytochrome *c* was prepared by the method described by Hettinger & Harbury (1964). Protein concentrations were determined by using the following

extinction coefficients: horse ferrocytochrome *c*, $\varepsilon_{550} = 27600 M^{-1} \cdot cm^{-1}$ (Schejter *et al.*, 1963); ferrocytochrome c_{551}, $\varepsilon_{551} = 28300 M^{-1} \cdot cm^{-1}$ (Horio *et al.*, 1960); azurin, $\varepsilon_{625} = 3500 M^{-1} \cdot cm^{-1}$ (Brill *et al.*, 1968). The extinction coefficients for *C. hippurus* and *D. discoideum* cytochromes *c* were taken to be the same as that for horse cytochrome *c*. Sodium ascorbate and ascorbic acid were from Sigma.

Experiments were performed in 0.1M-sodium tetraborate buffer, the pH of which was regulated by the addition of either HCl or NaOH.

Stopped-flow experiments were performed in a Durrum-Gibson instrument with a 2cm light path and a dead time of 3ms.

Regression analysis was performed with a standard least-squares programme on a PDP 10 computer.

Results

Effect of pH on the rate of reduction of redox proteins

Rapid-mixing experiments in which redox proteins were mixed with sodium ascorbate led to the reduction of the metal centres associated with the proteins. Over the pH range explored the progress curves for the reduction of the redox centres conformed to simple exponential curves. At pH values above neutrality the reduction of eukaryote cytochrome *c* was followed, as expected (Greenwood & Palmer, 1965; Wilson & Greenwood, 1971) by a slower process reflecting conformation changes in the molecule. Such slow processes were absent from the reduction kinetics of azurin and cytochrome c_{551} (Al-Ayash & Wilson, 1977). The rates of reduction were, in all cases, linearly dependent on ascorbate concentration, in agreement with previous reports (Greenwood & Palmer, 1965), allowing the apparent second-order rate constants for the reduction of these proteins by ascorbate to be determined. Fig. 1 shows the dependence of first-order rate constant on pH for a number of proteins. It is clear that there is a strong pH-dependence and that the basic shape of the curve is independent of the nature of the protein. The pH dependences shown in Fig. 1(*a*) are in broad agreement with those found for mammalian cytochrome *c* and mammalian cardiac cytochrome c_1 in their reactions with ascorbate (Greenwood & Palmer, 1965; Kaminsky *et al.*, 1975).

On the basis of the model given in the introduction, and assuming that ionization is fast compared with reduction, we may derive the following equation:

$$k_{app.} = \frac{k_1}{1 + [H^+]/K} \quad (3)$$

where $k_{app.}$ is the apparent second-order rate constant for the reduction of the redox proteins, k_1 is the true pH-independent second-order rate constant and K is the equilibrium constant for the ionization of ascorbate to form the ascorbate^{2-} species. Under the experimental conditions chosen, i.e. where $[H^+] > K$, we may simplify eqn. (3) to obtain eqn. (4):

$$\log k_{app.} = \log k_1 + \log K + pH \quad (4)$$

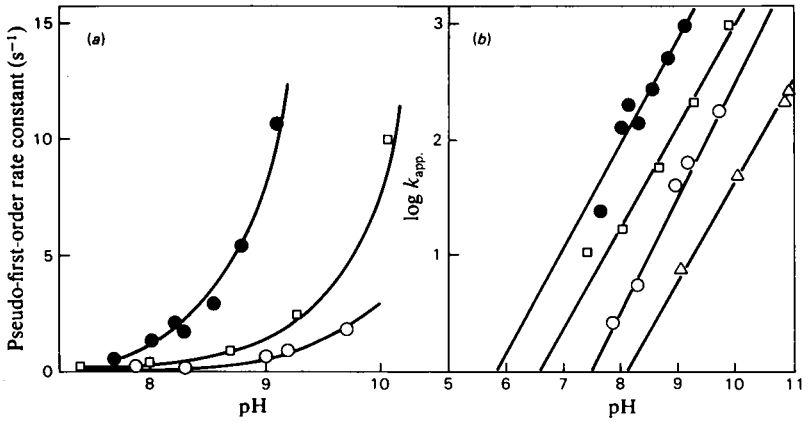

Fig. 1. *Effect of pH on the rate of ascorbate reduction of redox proteins*

(*a*) Pseudo-first-order rate constants for ascorbate reduction. ●, *C. hippurus* ferricytochrome *c*; □, *P. aeruginosa* cytochrome c_{551}; ○, azurin. The systems contained a few micromoles of each protein and 10mM-ascorbate in 0.1M-borate buffer. The temperature was 24°C. The wavelengths at which the reactions were followed in the stopped-flow apparatus were 417nm and 625nm for ferricytochrome *c* and azurin respectively. (*b*) The logarithmic plot of the second-order rate constants for the reduction of the above proteins as a function of pH together with that of cardiac cytochrome c_1. △, Rate data taken from Kaminsky *et al.* (1975). For clarity the data for cardiac cytochrome c_1 are displaced by 1 pH unit towards higher pH values.

According to eqn. (4) a plot of log $k_{app.}$ versus pH should yield a straight line of slope unity. Fig. 1(*b*) shows such a plot of the data from Fig. 1(*a*) together with the data for cardiac cytochrome c_1. Similar results were obtained for guanidinated horse heart cytochrome *c*.

It is clear that linear behaviour is observed in all cases and, moreover, that the slopes of these lines, irrespective of the protein under study, are close to the predicted value of unity. This result is consistent with the model given in eqns. (1) and (2). The characteristic shape of the curves in Fig. 1(*a*) is basically similar to that obtained by Kaminsky *et al.* (1975). The interpretation of this behaviour presented by these authors emphasized the existence of an inverse relationship between the enhanced rate of reduction by ascorbate and the pH-induced disappearance of the 695nm band (pK~9.2) and that this enhancement was a consequence of the increased accessibility of the haem iron to ascorbate. The finding that essentially similar behaviour is exhibited by bacterial cytochrome c_{551} in which pH-dependent bleaching of the 695nm absorption band occurs with pK>10 (Vinogradov, 1970), and by azurin, a non-haem blue copper protein, weakens this conclusion as does the fact that loss of the 695nm absorption band of cytochrome *c* leads to an ascorbate irreducible form of cytochrome *c* rather than a more reactive species (Wilson & Greenwood, 1971). Thus, although in principle ionization of groups with high pK_a values on the proteins could lead to changes at the redox centres resulting in the kinetic behaviour depicted in Fig. 1, we believe that as all redox proteins examined behave in an essentially similar fashion irrespective of origin, nature of the metal and presence or absence of redox-linked ionizations in the pH range explored (cf. cytochrome *c* and cytochrome c_{551}) the pH dependences observed are due to ionization of the reducing agent. These findings therefore lend support to the model presented earlier, i.e. that the observed changes are not due to the protein itself, but due to the fact that ascorbic acid exists in two forms in pH-dependent equilibrium and that ascorbate^{2-} is the attacking species, where at high pH values the concentration of this species is increased.

By applying eqn. (4), and with the knowledge of the pK value of ascorbate it is possible to derive from the intercepts of Fig. 1(*b*) the true rate constants (k_1) for the reduction of the redox proteins by ascorbate^{2-} species. In Table 1 the values obtained for k_1 for a number of redox proteins are listed together with the values of their isoelectric points.

In an attempt to eliminate the possibility that superoxide radicals ($O_2^{-\cdot}$), formed from the oxidation of ascorbate by molecular oxygen, were involved in the reduction of redox proteins, we have undertaken a series of experiments in which horse heart cytochrome *c* was reduced by ascorbate either under anaerobic conditions or in the presence of superoxide dismutase (20μM). The results of these experiments were compared with those of control experiments performed in the presence of oxygen and without the addition of superoxide dismutase. It was clear that in all cases the progress curves were identical and thus allowed us to exclude ($O_2^{-\cdot}$) radicals from the reduction mechanism.

Table 1. *Second-order rate constants for the reduction of redox proteins by the ascorbate^{2-} ion*

Protein	k_1($M^{-1}\cdot s^{-1}$)	pI
Horse heart cytochrome *c*	3.1×10^5	10.5
Guanidinated horse heart cytochrome *c*	1.1×10^5	11.2
Dictyostelium discoideum cytochrome *c*	3.9×10^5	10.2
Coryphaena hippurus cytochrome *c*	2.0×10^5	10.3
Bovine cardiac cytochrome c_1	1.0×10^4	5.5
Pseudomonas aeruginosa cytochrome c_{551}	2.9×10^4	4.7
Pseudomonas aeruginosa azurin	1.0×10^4	5.5

Temperature-dependences

Fig. 2 shows the effect of temperature on the rate of reduction of azurin and cytochrome c_{551} at a number of pH values. The parallel nature of the logarithmic plots in Fig. 2 indicates that the apparent activation energy for reduction of these proteins by ascorbate is pH-independent.

Application of eqn. (4) to each of these linear plots allows us to estimate the value of the quantity ($\log k_1+\log K$) at a number of temperatures.

The temperature dependence of log$K$ was determined by performing pH titrations of ascorbic acid in the range pH 9.0–12.5. The second pK of ascorbate was found to decrease with increasing temperature and a Van't Hoff plot of data yielded a value of 38.5kJ/mol for the enthalpy of ionization.

By combining the temperature-dependences of ($\log k_1+\log K$) and of log$K$ the variation of the second-order rate constant for reduction (k_1) with temperature could be obtained. As an example, Fig. 3 shows an Ahrrenius plot for azurin derived from the data in Fig. 2. The values of the activation parameters for the reduction (ΔH^* and ΔS^*) were determined by fitting the best straight line to the data points in Fig. 3 by regression analysis. These values are given in Table 2 together with those determined in a similar manner for a number of other redox proteins.

Reaction of cytochrome c with imidazole

Azide, cyanide and imidazole can co-ordinate to the haem iron of cytochrome *c* (George *et al.*, 1967)

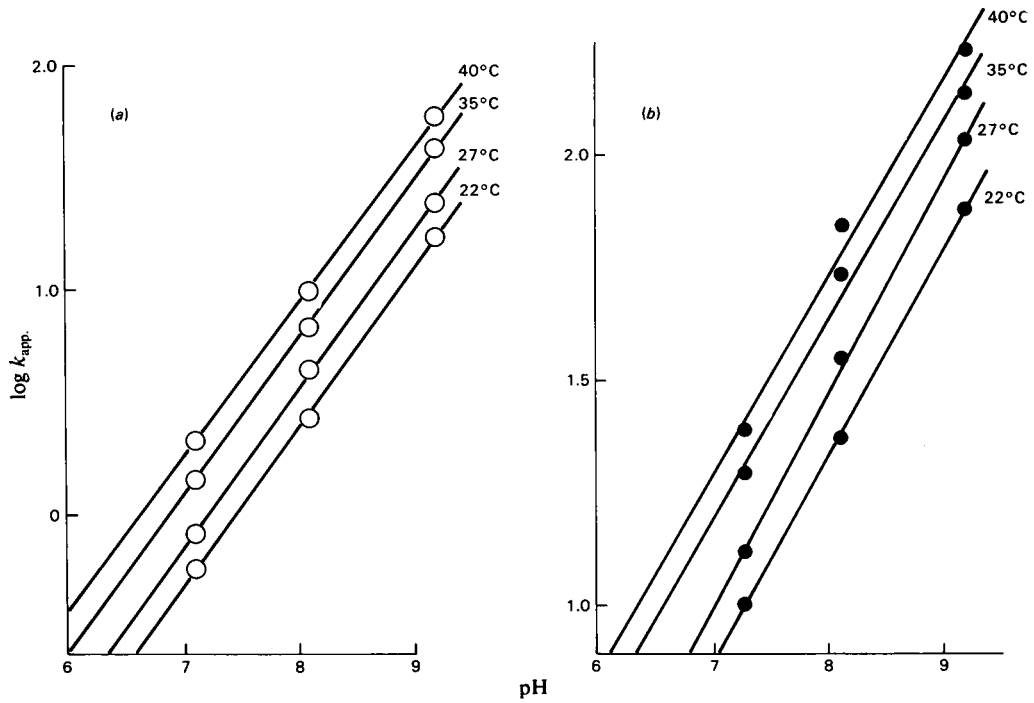

Fig. 2. *Temperature-dependence of $k_{app.}$ for azurin and cytochrome c_{551}*
Dependence of the logarithm of the apparent second-order rate constant for the reduction of azurin (*a*) and cytochrome c_{551} (*b*) by ascorbate as a function of temperature and pH. Reduction by 20mM-ascorbate was followed at either 625nm for azurin or 417nm for cytochrome c_{551}. The buffer was 0.1M-borate.

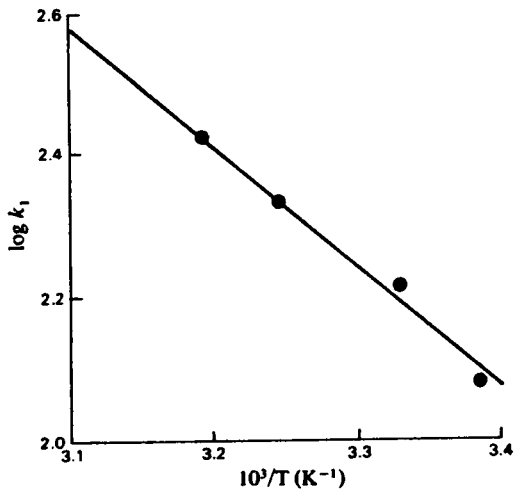

Fig. 3. *Temperature-dependence of the true second-order rate constant (k_1) for reduction of azurin by ascorbate*
k_1 was determined at each temperature from the knowledge of $k_{app.}$ and the pK of ascorbic acid at each temperature (see the text). ——, Best straight line fitted by regression analysis.

and evidence suggests that these ligands bind by displacing methionine (Schejter & Aviram, 1969). Sutin & Yandell (1972) studied the binding of imidazole to ferricytochrome *c* at pH 7.0, concluding that imidazole displaces the sulphur of methionine from the 6th co-ordination position of the haem and placed an upper limit for the rate constant for this displacement at $60s^{-1}$. The binding of imidazole to the haem iron is thus rate limited by the 'off' rate of methionine from the central iron atom.

Yandell *et al.* (1973) and Creutz & Sutin (1973) have reported that reducing agents such as Cr(II), SO_2^- and $S_2O_4^{2-}$ are unable to reduce cytochrome *c* at rates in excess of $60s^{-1}$ at pH7.0. On this basis these authors conclude that these reductants transfer electron to the iron atom of ferricytochrome *c* by an 'inner-sphere' mechanism.

In order to ascertain whether the same situation applied to reduction by ascorbate^{2-} we have performed similar experiments. We have chosen to undertake these experiments at alkaline pH where the reduction of cytochrome *c* is fast [due, according to the model in eqn. (1), to the increased concentration of the ascorbate^{2-} species] and where any rate limit can easily be detected.

Table 2. *Rate constants for electron transfer reactions*
Ligand abbreviations: py, pyridine; phen, 1,10-phenanthroline.

Protein	Reductant or oxidant	k_1 ($M^{-1}\cdot s^{-1}$)	ΔH^* (kJ/mol)	ΔS^* ($J\cdot mol^{-1}\cdot K^{-1}$)	Reference
Azurin II	$Ascorbate^{2-}$	1.0×10^4	12.9 ± 1.7	-160 ± 5.5	Present work
Cytochrome c_{551} III	$Ascorbate^{2-}$	2.9×10^4	1.51 ± 2.43	-178 ± 8	Present work
Cytochrome *c* III (horse heart)	$Ascorbate^{2-}$	3.1×10^5	11.51 ± 1.26	-92.5 ± 4	Present work
Cytochrome *c* III (horse heart)	$Fe[EDTA]^{2-}$	2.57×10^4	25.1	−75	Hodges *et al.* (1974)
Cytochrome *c* III (horse heart)	$[Ru(NH_3)_6]^{2+}$	3.8×10^4	12.1	−117	Ewall & Bennett (1974)
Cytochrome *c* II (horse heart)	$[Ru(NH_3)_5py]^{3+}$	1.86×10^4	35.1	−46	Cummins & Gray (1977)
Azurin I	$[Ru(NH_3)_5py]^{3+}$	2×10^3	36.8	−58.5	Cummins & Gray (1977)
Cytochrome *c* II	$[Co(phen)_3]^{3+}$	1.5×10^3	47.3	−26	McArdle *et al.* (1974)

The rate of binding of imidazole to ferricytochrome *c* at pH9.0 was determined by rapidly mixing the two reagents in a stopped flow apparatus. Binding of imidazole to the central iron atom induces a small blue shift in the soret peak of cytochrome *c* and also perturbs the spectrum in the visible region (Schejter & Aviram, 1969). The kinetics of binding were followed by monitoring the spectral change at 410nm.

At all concentrations of imidazole used, and under pseudo-first-order conditions, the binding reaction was found to conform to a simple exponential decay process. This finding is somewhat surprising, considering that at pH9 two forms of the protein exist in solution. These differ, in that one form possesses methionine (cytochrome *c* III_S) and the other probably the amino group of lysine-79 as the 6th haem ligand (cytochrome *c* III_N) (Gupta & Koenig, 1971) and it is unlikely that these two ligands would have identical 'off' rates. A solution to this problem may be that no spectral change is engendered on replacing the nitrogen of lysine-79 by the nitrogen of an added imidazole group and thus this displacement is not seen spectrophotometrically. It is clear from Fig. 4 that the binding of imidazole is rate limited, presumably by the rate of methionine displacement at a value of approx. $8\,s^{-1}$, significantly lower than the corresponding rate measured by Sutin & Yandell (1972) at pH7.0. Greenwood & Palmer (1965) report a value of $0.49\,s^{-1}$ for this process over the concentration range 5–50mM-imidazole, the comparable values from Fig. 4 are $1.4–2.4\,s^{-1}$ over the same concentration range.

Parallel experiments conducted at pH9.0 and monitoring the reduction of ferricytochrome *c* by high concentrations of ascorbate showed that the reduction of ferricytochrome *c* by $ascorbate^{2-}$ may occur at rates far in excess of $8\,s^{-1}$ (rates in excess of $100\,s^{-1}$ were obtained with 0.4M-ascorbate). These results indicate that reduction does not proceed by an inner-sphere mechanism after methionine displacement and thus argue for an outer-sphere mechanism.

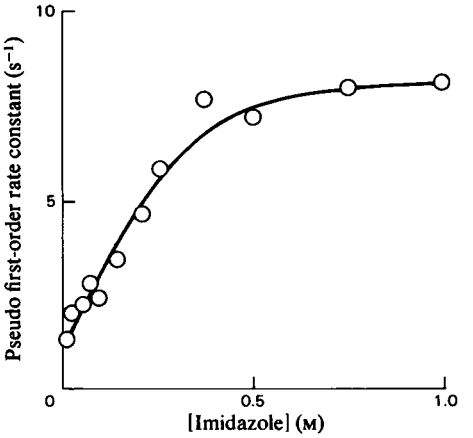

Fig. 4. *Imidazole concentration dependence of the rate of binding of imidazole to ferricytochrome c*
A solution of 7.5μM-horse heart cytochrome *c* was mixed with solutions containing known concentrations of imidazole in a stopped-flow apparatus. The buffer was 0.1M-sodium borate, pH 9.0. The reaction was followed at 410nm. The temperature was 21°C.

The kinetic experiments designed to measure the upper limit for the rate of binding of imidazole to ferricytochrome *c* at pH9.0 also allow us to determine the binding constant of imidazole to cytochrome *c* at this pH. Under pseudo-first-order conditions and where the exchange rate of the intrinsic methionine ligand is rapid compared with imidazole binding, i.e. at low imidazole concentrations, the first-order rate for the approach to equilibrium on mixing imidazole with cytochrome *c* is given by:

$$k = k_{off} + k_{on}\,[\text{imidazole}] \qquad (5)$$

where k is the measured rate, k_{off} is the rate constant for dissociation of imidazole from the haem and k_{on} is the combination rate constant of imidazole and cytochrome *c*. This latter rate is a function of the intrinsic combination constant of imidazole with the

ferric haem and the binding constant of the intrinsic methionine ligand to the central iron. From the slope of the low imidazole concentration region of Fig. 4 we may derive an apparent second-order rate constant for binding imidazole to ferricytochrome *c*. This value is $22\,M^{-1} \cdot s^{-1}$. The intercept of the same Figure gives a value of $1.2\,s^{-1}$ for the dissociation constant of imidazole from the iron. The overall binding constant is thus the ratio of these values, i.e. $K = 20\,M^{-1}$ (approximately). By monitoring the total absorbance changes resulting from mixing ferricytochrome *c* with different concentrations of imidazole in the stopped-flow apparatus we may construct a binding curve. Fig. 5 shows the binding curve obtained. The solid line shows a theoretical fit to the experimental points using a binding constant, $K = 28.6\,M^{-1}$, in fair agreement with the kinetically determined value. This result is in good agreement with the value of $29\,M^{-1}$ (at 18°C) reported by Schejter & Aviram (1969).

Discussion

Ascorbic acid has been reported to form radicals in solution. However, Bielski *et al.* (1975) have established that any such radicals formed react only slowly with ferricytochrome *c* and decay mainly by disproportion. It is unlikely therefore that under the conditions of our experiments such radicals play any important role in the reduction of redox proteins, although they may be intermediates in the overall mechanism of reaction (see eqn. 6). This situation may be contrasted with that in which ascorbic acid reduces cytochrome *c* in the presence of ascorbic acid oxidase and molecular oxygen. Under these circumstances enzyme-generated ascorbic acid radicals appear to play the major role in reducing the haem protein (Yamazaki, 1962). Similarly $O_2^{-\cdot}$ radicals formed in solution may also be excluded from the reduction mechanism by reference to the fact that neither changes in the oxygen concentration nor the presence of superoxide dismutase have any effect on the reduction of ferricytochrome *c* by ascorbate. In addition it has been reported that ascorbate is a scavenger for any $O_2^{-\cdot}$ radicals that may be present (Halliwell & Foyer, 1976; Allen & Hall, 1973; Nishikimi, 1975).

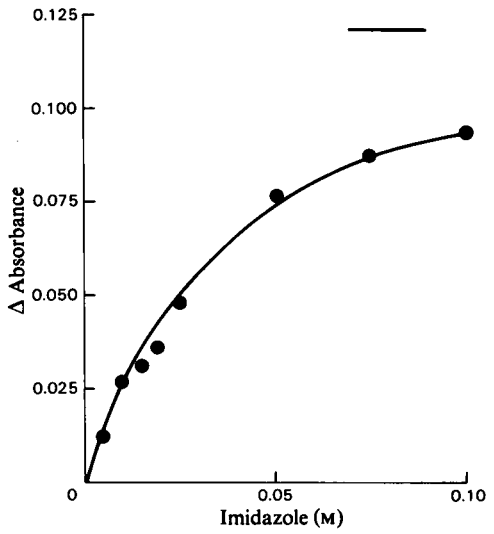

Fig. 5. *Saturation curve for the binding of imidazole to ferricytochrome c*

The total absorbance changes resulting from mixing 7.5 μM-horse heart cytochrome *c* with known concentrations of imidazole in 0.1 M-borate buffer, pH 9.0, were measured at 410 nm in a stopped-flow apparatus. The horizontal line denotes the absorbance change expected on fully saturating ferricytochrome *c* with imidazole. The temperature was 21°C.

At pH values around neutrality ascorbic acid exists mainly as the H–ascorbate$^-$ ion. Weissberger *et al.* (1943), however, demonstrated that at neutral pH and above the autoxidation of ascorbate with molecular oxygen proceeds via the ascorbate^{2-} species and that the rate of autoxidation increases as the ascorbate^{2-} ion concentration increases with pH.

The above discussion and the results presented argue for the ascorbate^{2-} species being the reductant for redox proteins acting as single electron acceptors. This conclusion is in agreement with the findings of Yamazaki (1962), who concluded that in non-enzymic systems it is the doubly dissociated ascorbic acid ion that reduces cytochrome *c*.

A mechanism incorporating ascorbate^{2-} as a single-electron reducing agent may be formulated in eqns. (6), (7) and (8):

$$\text{Ascorbate}^{2-} + M^{n+} \rightarrow M^{(n-1)+} + \text{ascorbate}^{-\cdot} \quad (6)$$

$$2\,\text{Ascorbate}^{-\cdot} \rightarrow \text{ascorbate}^{2-} + \text{ascorbate}^{2\cdot} \quad (7)$$

$$\text{Ascorbate}^{2\cdot}{}_N \rightarrow \text{dehydroascorbate} \quad (8)$$

Where M^{n+} and $M^{(n-1)+}$ are the oxidized and reduced states of the metal redox centres. Reduction of the ferric haem of the cytochromes or the copper atom of azurin leads to the production of the anionic ascorbate radical, which decays by a slow disproportionation reaction (Bielski *et al.*, 1975) in which one ascorbate^{2-} ion is produced together with a diradical which in turn rapidly rearranges to produce the known product of ascorbate oxidation, namely dehydroascorbate.

The overall stoicheiometry of the reaction is thus:

$$\text{Ascorbate}^{2-} + 2M^{n+} \rightarrow 2M^{(n-1)+} + \text{dehydroascorbate}$$

The linearity of the results given in Fig. 1(*b*) down to pH 7 implies that even at pH values around neutrality it is the ascorbate^{2-}, comprising only approx. 10^{-2}% of the total ascorbic acid present, which acts

as the major reductant for the redox proteins studied. Species such as H–ascorbate$^-$ may play a minor role as reductants near neutrality and it is possible that deviation of our data in Fig. 1(*b*) from slopes of unity may result from such complications not explicitly accounted for in the model of eqns. (1) and (2).

Where ascorbate is used as a reducing agent for cytochrome *c* in steady-state investigations of cytochrome oxidase the concentration of this reductant (ascorbate–H_2) can enter explicitly into the steady-state equation describing the kinetic model (Minnaert, 1961; Petersen *et al.*, 1976). Although the general conclusions drawn from such studies may remain valid it is clear that quantitative calculations would be sensitive to the substitution of [ascorbate^{2-}] for [ascorbate–H_2].

Mechanism of electron transfer from ascorbate^{2-} to a single-site redox protein

Reduction rates in excess of the ligand-replacement rate for cytochrome *c* (Fig. 4) suggest that ascorbate^{2-} is transferring electrons to the central haem iron by an outer-sphere mechanism. Such a mechanism would probably involve electron transfer from the ascorbate^{2-} ions to the exposed edge of the haem group. Studies by Hodges *et al.* (1974) and McArdle *et al.* (1974) have shown that a number of inorganic reductants or oxidants transfer electrons to and from the iron of cytochrome *c* via the haem edge. Structural data indicate that the haem 'crevice' of both eukaryote and prokaryote cytochromes *c* have, at neutral pH, a positively charged rim composed of six evolutionary invariant amino acid residues. In addition there is a negative charge at the top of the crevice, an aromatic group to the bottom and to one side and a glutamine residue, offering hydrogen-bonding possibilities to the other (Dickerson & Timkovich, 1975). There is therefore considerable scope for protein–ascorbate^{2-} interaction in the vicinity of the haem edge. Table 1 shows that those proteins which carry an overall positive charge under the conditions of the experiment, as judged from their pI values, have a significantly higher rate of reduction by the ascorbate^{2-} ion than those which bear an overall negative charge. Although the overall charge on the protein is unlikely to be the major factor governing the rate of reaction with ascorbate (it is possible that an overall negatively charged protein may nevertheless contain a positively charged centre) we may speculate that electrostatic interactions between the residues around the redox site and this reductant may play a role in determining the rate of the electron-transfer reaction.

Support for the view that an outer-sphere mechanism is operating in the reduction of redox proteins by ascorbate^{2-} ion is given in Table 2, where the rate constants and activation parameters for electron transfer from ascorbate^{2-} are compared with like parameters obtained for known outer-sphere oxidants and reductants. The values determined for the reduction of redox proteins by ascorbate^{2-} are in reasonable agreement with those values for the known outer-sphere reactants, i.e. we see rate constants of the order of 10^4–$10^5\,M^{-1}\cdot s^{-1}$, and a small positive enthalpy and a negative entropy of activation.

From these comparisons it is apparent that no special 'protein effect' such as conformational change need be invoked to explain the activation parameters for ascorbate^{2-} and the above redox proteins reactions. The observed $\Delta H^{\ddagger}$ values are consistent with an activation process involving only minor rearrangements in the co-ordination environments of the iron or the copper atoms in these proteins.

A difficulty that arises from the proposed model of the reduction of ferricytochrome *c* by ascorbate^{2-} is that no reason is given to account for the fact that ascorbate^{2-} does not reduce the isomer of cytochrome *c* which exists at alkaline pH (cytochrome *c* III_N). It has been suggested that this isomer differs from native cytochrome *c* in that the sulphur atom of methionine-80 is replaced by a nitrogen atom (possibly of lysine-79). However, it is unlikely that such a ligand exchange would result in the haem edge no longer being exposed and thus electron transfer via this edge should still be possible. One reason may be that as the redox potential of the alkaline isomer is significantly lower than that of the native molecule (Margalit & Schejter, 1973) reduction does not occur for thermodynamic rather than kinetic reasons. There is some difficulty in judging the likelihood of this explanation given that there exists uncertainty over the value of the redox potential of the ascorbate^{2-}/dehydroascorbate couple.

Alternatively, and perhaps more likely, is the proposal that ascorbate does in fact reduce the alkaline form of cytochrome *c* [as does Fe(EDTA)$^{2-}$; Hodges *et al.*, 1974], but does so at a rate significantly slower than the rate of isomerization between cytochrome III_N and cytochrome III_S. In these circumstances it would appear that the cytochrome III_N form of the protein was ascorbate irreducible.

As ascorbate appears from our results to act as an outer-sphere reductant for cytochrome *c* we may get some indication of the relative rates of reduction of the two forms of this protein by application of the simple Marcus (1963) theory, which relates the rate constant for electron transfer between two different redox sites (k_{12}) to the equilibrium constant (K_{12}) for this reaction and the self-exchange rates for the individual components (k_{11} and k_{22}), i.e. $k_{12} = (k_{11}k_{22}K_{12})^{\frac{1}{2}}$.

Taking the redox potential of ascorbate as −0.012 V (pH 8.7, Sober, 1970) and those of cytochrome *c* III_S as 0.261 V and cytochrome *c* III_N as 0.09 V (Margalit & Schejter, 1973) we may conclude that the reduction

of cytochrome III_N will be at least two orders of magnitude slower than the reduction of cytochrome III_S. As we typically measure rates of approx. 10–20 s^{-1} for the reduction of cytochrome *c* III_S at pH9.0 (see Fig. 1*a*) then we would expect the rate of reduction of cytochrome III_N under the same conditions to be less than 0.1 s^{-1}. This rate is of the same order as the rate of isomerization (Wilson & Greenwood, 1971) and may be considerably less than this if the self-exchange rate (k_{11}) of cytochrome III_N is less than that for cytochrome III_S. In this way the reduction of cytochrome *c* III_N by ascorbate would be masked and one would be drawn to the conclusion that this form of the protein is indeed irreducible by ascorbate.

We thank Mr. J. Masiakowski for skilful technical assistance.

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