KINETICS AND MECHANISM OF THE REACTION BETWEEN OXIDIZED CYTOCHROME c AND ASCORBIC ACID

Joaquin F. PEREZ-BENITO* and Conchita ARIAS

Departamento de Quimica Fisica, Facultad de Quimica, Universidad de Barcelona, Marti i Franques 1, 08028-Barcelona, Spain

Received April 13, 1990
Accepted June 5, 1990

The reaction between horse-heart cytochrome c and ascorbic acid has been investigated in the pH range 5·5—7·1 and at 10·0—25·0°C. The rate shows a first-order dependence on the concentration of cytochrome c, it increases in a non-linear way as the concentration of ascorbic acid increases, it increases markedly with increasing pH and, provided that the ionic strength of the medium is high enough, it fulfills the Arrhenius equation. The apparent activation energy increases as the pH of the solution increases. The results have been explained by means of a mechanism that includes the existence of an equilibrium between two forms (acidic and basic) of oxidized cytochrome c: $cyt\text{–}H^{+}\text{–}Fe^{3+} + OH^{-} \rightleftarrows cyt\text{–}Fe^{3+} + H_2O$, whose equilibrium constant is $(6{\cdot}7 \pm 1{\cdot}4) \cdot 10^{8}$ at 25·0°C, the acidic form being more reducible than the basic one. It is suggested that there is a linkage of hydrogenascorbate ion to both forms of cytochrome c previous to the redox reactions. Two possibilities for the oxidant–reductant linkage (binding and adsorption) are discussed in detail.

Both cytochrome c and ascorbic acid are biologically important substances whose physiological activity resides largely in their redox behaviour[1,2]. Because of this, the reaction between cytochrome c (oxidant) and ascorbic acid (reductant) has attracted some interest for several years[3-11]. However, despite the extensive study made on this reaction, its mechanism is not completely understood[5]. Moreover, although the reaction between cytochrome c and excess ascorbic acid under slightly acid or neutral conditions is slow enough to allow to be followed with a conventional spectrophotometer, almost all the kinetic studies published on the reaction were done using stopped-flow technique. As a result, most of the kinetic data available concerning the cytochrome c — ascorbic acid reaction were obtained under conditions in which the reaction takes place very rapidly.

Now, we have studied the kinetics of the reaction with the aid of a conventional spectrophotometer, under conditions in which the reaction is relatively slow.

EXPERIMENTAL

Horse-heart cytochrome c was purchased from Sigma (Type III). All the other chemicals (ascorbic acid, potassium dihydrogen phosphate, dipotassium hydrogen phosphate and potassium

chloride) were purchased from Merck (analytical grade reagents). The solvent (water) was redistilled in an all-glass apparatus (the first time from a potassium permanganate solution).

The kinetic runs were done under constant-pH conditions (KH_2PO_4–K_2HPO_4 buffer) and with a strong excess of ascorbic acid in respect to oxidized cytochrome c (100 to 500-fold). The formation of reduced cytochrome c was followed measuring its absorbance at 550 nm with a Varian Cary 219 UV-VIS spectrophotometer. Quartz cuvettes (pathlength 1 cm) were thermostated throughout the runs.

RESULTS

Determination of rate constants. Under the experimental conditions, the reaction followed the integrated rate law:

$$\ln (A_\infty - A) = \ln (A_\infty - A_0) - k_1 t \,, \qquad (1)$$

where A is the absorbance at time t, its initial and final values being A_0 and A_∞, respectively, whilst k_1 is the pseudo-first order rate constant. The runs were followed until 80% of the reaction was completed; then, the solutions were left in the thermostated cuvette of the spectrophotometer till A_∞ was reached. The average linear correlation coefficient associated with Eq. (*1*) for 195 experiments was 0·9997 ± 0·0004. A typical pseudo-first order plot is shown in Fig. 1. For each kinetic parameter 2–4 determinations were made, and the values given in Figs 2–5 and Tables I–III are the corresponding averages (in the tables the standard deviations are also given).

Dependence on oxidant concentration. As can be seen in Table I, the initial rate was directly proportional to the initial concentration of cytochrome c (the other

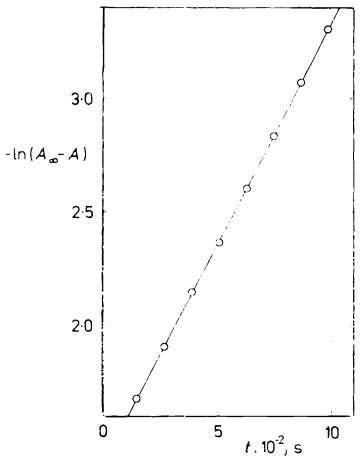

FIG. 1

Pseudo-first order plot. Experimental conditions: $[\text{cytochrome c}]_0 = 1{\cdot}60 \,.\, 10^{-5}$ mol . . l^{-1}, $[\text{ascorbic acid}] = 1{\cdot}60 \,.\, 10^{-3}$ mol l^{-1}, pH 5·54, ionic strength 2·24 mol l^{-1} (KCl) and temperature 25·0°C

experimental conditions remaining constant). This confirms the situation made evident by Fig. 1; i.e., the reaction is first order as far as the oxidant is concerned.

Dependence on reductant concentration. A similar first-order behaviour was not found for the reductant, since the k_1 values did not increase linearly with the ascorbic acid concentration. On the contrary, plots of the types $1/k_1$ vs $1/[\text{ascorbic acid}]$ and $\log k_1$ vs $\log [\text{ascorbic acid}]$ were found to be linear at various temperatures (see Figs 2 and 3, respectively).

Dependence on pH. The rate of the reaction was found to increase markedly as the pH of the solution increased at all the temperatures studied. The pseudo-first order rate constant seemed to depend on hydrogen ion concentration according to the relation

$$k_1(a + [H^+]) = b + c/[H^+] . \qquad (2)$$

Parameter a was calculated from linear plots of $k_1(a + [H^+])$ vs $1/[H^+]$ (Fig. 4). The variation of parameter a in the interval 10·0–25·0°C was within the experimental error margin, its average value being $a = (6{\cdot}8 \pm 1{\cdot}4) . 10^{-6}$ mol l^{-1}.

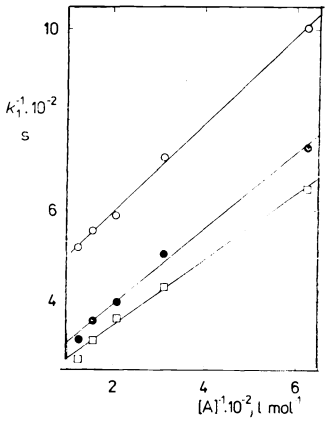

FIG. 2

Dependence of the pseudo-first order rate constant on the reductant concentration at various temperatures (double reciprocal plots). Experimental conditions: $[\text{cytochrome c}]_0 = 1{\cdot}60 . 10^{-5}$ mol l^{-1}, $[A] \equiv$ $\equiv [\text{ascorbic acid}] = (1{\cdot}60-8{\cdot}01) . 10^{-3}$ mol . l^{-1}, pH 5·61, ionic strength 1·22 mol l^{-1} (KCl) and temperature 10·0 (○), 15·0 (●) and 20·0 (□) °C

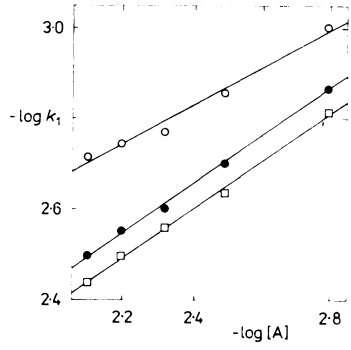

FIG. 3

Dependence of the pseudo-first order rate constant on the reductant concentration at various temperatures (double logarithmic plots). Experimental conditions: $[\text{cytochrome c}]_0 = 1{\cdot}60 . 10^{-5}$ mol l^{-1}, $[A] \equiv$ $\equiv [\text{ascorbic cid}] = (1{\cdot}60-8{\cdot}01) . 10^{-3}$ mol . l^{-1}, pH 5·61, ionic strength 1·22 mol l^{-1} (KCl) and temperature 10·0 (○), 15·0 (●) and 20·0 (□) °C

Dependence on ionic strength. The effect of ionic strength of the medium on rate of the reaction was studied by means of additions of potassium chloride to the solutions. At all the temperatures studied, the reaction was markedly slowed down by additions of potassium chloride; however, the decrease in the rate was due (at least partially) to a decrease in the pH of the solution caused by the addition of electrolyte. The k_1 values obtained were corrected in order to discount the change in k_1 due to the change in pH, and the results (referred to a constant pH) are given in Table II. Whereas at 10·0°C k_1 was fairly independent of the KCl concentration, a smooth decrease in k_1 with rising concentration of electrolyte was observed at 15·0°C; finally, a definite decrease in k_1 was observed at 20·0°C when the KCl concentration changed from 0·0 to 0·5 mol l^{-1} although further increases in the electrolyte concentration resulted in a slight increase in k_1.

Dependence on temperature. The reaction between oxidized cytochrome c and ascorbic acid fulfilled the Arrhenius equation only under certain experimental conditions. For instance, from the data given in Table II it follows that at low ionic strengths the fit is much worse than at high ionic strengths. Consequently, the deter-

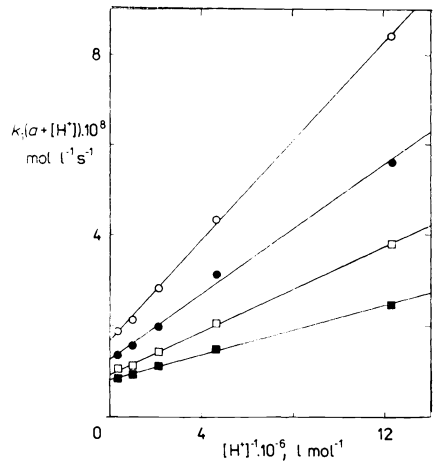

FIG. 4

Dependence of the pseudo-first order rate constant on the hydrogen ion concentration at various temperatures. Experimental conditions: [cytochrome c]$_0$ = 1·60 . 10^{-5} mol . l^{-1}, [ascorbic acid] = 1·60 . 10^{-3} mol l^{-1}, pH 5·54—7·09, ionic strength 2·34 mol l^{-1} (KCl) and temperature 10·0 (■), 15·0 (□), 20·0 (●) and 25·0 (0) °C

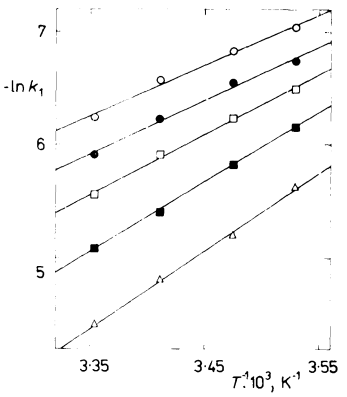

FIG. 5

Arrhenius plots at various pH values. Experimental conditions: [cytochrome c]$_0$ = = 1·60 . 10^{-5} mol l^{-1}, [ascorbic acid] = = 1·60 . 10^{-3} mol l^{-1}, ionic strength 2·34 mol l^{-1} (KCl), temperature 10·0 to 25·0°C and pH 5·54 (○), 6·00 (●), 6·33 (□), 6·67 (■) and 7·09 (△)

TABLE I

Effect of cytochrome c concentration on the initial rate. Experimental conditions: [ascorbic acid] = $1{\cdot}60 \cdot 10^{-3}$ mol l^{-1}, pH 5·55, ionic strength 2·11 mol l^{-1} (KCl) and temperature 15·0°C. The pseudo-first order rate constant was calculated as: $k_1 = r_0/[\text{cytochrome } c]_0$

[cytochrome $c]_0 \cdot 10^5$ mol l^{-1}	$r_0 \cdot 10^8$ mol l^{-1} s^{-1}	$k_1 \cdot 10^3$ s^{-1}
0·92	1·03 ± 0·02	1·12 ± 0·02
1·25	1·38 ± 0·03	1·10 ± 0·02
1·59	1·72 ± 0·03	1·08 ± 0·02
1·92	2·08 ± 0·06	1·08 ± 0·03
2·26	2·44 ± 0·07	1·08 ± 0·03

TABLE II

Dependence of the pseudo-first order rate constant on the potassium chloride concentration at various temperatures. Experimental conditions: [cytochrome $c]_0 = 1{\cdot}60 \cdot 10^{-5}$ mol l^{-1}, [ascorbic acid] = $1{\cdot}60 \cdot 10^{-3}$ mol l^{-1}, pH 5·76 and ionic strength 0·19 mol l^{-1} (KCl). The k_1 values are given in 10^{-3} s^{-1}

[KCl] mol l^{-1}	k_1 at 10·0°C	k_1 at 15·0°C	k_1 at 20·0°C
0·00	0·99 ± 0·02	1·64 ± 0·02	2·11 ± 0·11
0·51	0·92 ± 0·07	1·45 ± 0·05	1·52 ± 0·02
1·02	1·07 ± 0·06	1·46 ± 0·11	1·59 ± 0·13
1·54	0·95 ± 0·04	1·34 ± 0·08	1·74 ± 0·02
2·05	0·90 ± 0·05	1·27 ± 0·06	1·67 ± 0·01

TABLE III

Apparent activation energies at various pH values. Experimental conditions: [cytochrome $c]_0$ = = $1{\cdot}60 \cdot 10^{-5}$ mol l^{-1}, [ascorbic acid] = $1{\cdot}60 \cdot 10^{-3}$ mol l^{-1}, ionic strength 2·34 mol l^{-1} (KCl) and temperature 10·0—25·0°C

pH	5·54	6·00	6·33	6·67	7·09
E_a, kJ mol^{-1}	37 ± 2	40 ± 3	44 ± 2	51 ± 1	57 ± 2

mination of the activation parameters was made at high concentration of potassium chloride (ionic strength 2·34 mol l^{-1}); under these conditions, the Arrhenius equation holds at all the pHs studied (Fig. 5). The corresponding apparent activation energies were calculated from the slopes of the $\ln k_1$ vs $1/T$ plots given in Fig. 5. Definite increase in the apparent activation energy with rising pH was found (Table III).

DISCUSSION

Mechanism

In slightly acidic or neutral solutions, the stoichiometry of the reaction can be represented as follows:

$$2\ \text{cyt–Fe}^{3+} + \text{AH}^- \rightarrow 2\ \text{cyt–Fe}^{2+} + \text{A} + \text{H}^+ , \qquad (3)$$

where cyt–Fe^{3+} and cyt–Fe^{2+} are the oxidized and reduced forms of cytochrome c, respectively, whilst AH^- stands for hydrogenascorbate ion (predominant) and A for dehydroascorbic acid.

As will be shown later, the kinetic parameters experimentally found in this work are consistent with the following mechanism for the reaction.

$$\text{cyt–H}^+\text{–Fe}^{3+} + \text{OH}^- \rightleftarrows \text{cyt–Fe}^{3+} + \text{H}_2\text{O} \qquad (4)$$

$$\text{cyt–H}^+\text{–Fe}^{3+} + \text{AH}^- \rightleftarrows \text{cyt–H}^+\text{–Fe}^{3+}\text{–AH}^- \qquad (5)$$

$$\text{cyt–Fe}^{3+} + \text{AH}^- \rightleftarrows \text{cyt–Fe}^{3+}\text{–AH}^- \qquad (6)$$

$$\text{cyt–H}^+\text{–Fe}^{3+}\text{–AH}^- + \text{OH}^- \rightarrow \text{cyt–Fe}^{2+} + \text{A}^{\bullet -} + \text{H}_3\text{O}^+ \qquad (7)$$

$$\text{cyt–Fe}^{3+}\text{–AH}^- + \text{OH}^- \rightarrow \text{cyt–Fe}^{2+} + \text{A}^{\bullet -} + \text{H}_2\text{O} \qquad (8)$$

$$\text{cyt–H}^+\text{–Fe}^{3+} + \text{A}^{\bullet -} \rightarrow \text{cyt–Fe}^{2+} + \text{A} + \text{H}^+ \qquad (9)$$

$$\text{cyt–Fe}^{3+} + \text{A}^{\bullet -} \rightarrow \text{cyt–Fe}^{2+} + \text{A} \qquad (10)$$

In Eq. (*4*) we have supposed that, under the slightly acidic or neutral conditions of this work, there are two forms of oxidized cytochrome c in equilibrium, one acidic (represented by cyt–H^+–Fe^{3+}) and the other basic (represented by cyt–Fe^{3+}), so that their concentrations are related by the relation

$$K_4 = [\text{BC}]/[\text{AC}]\,[\text{OH}^-] , \qquad (11)$$

where K_4 is the equilibrium constant associated with Eq. (*4*), whilst AC and BC stand, respectively, for the acidic and basic forms of oxidized cytochrome c. Actually, a similar hypothesis has already been used by other authors[3,4], and it has also been proved that there are several forms of oxidized cytochrome c in pH-related equilibrium[12,13].

In Eqs (*5*) and (*6*) the predominant form of the reductant becomes attached to the oxidant, either to the acidic form, Eq. (*5*), or to the basic one, Eq. (*6*). According to the pK_1 and pK_2 values for ascorbic acid[14,15], under the experimental conditions of this work (pH 5·54–7·09) the reductant is present essentially as hydrogenascorbate ion, AH^-, and, therefore, that is the form of the reductant that has to become attached to the oxidant. For the interpretation of Eqs (*5*) and (*6*) there are two distinct alternatives, which will be discussed in detail later.

It has been assumed that the hydrogenascorbate ion attached to the oxidant reacts with hydroxyl ion; again, there are two possibilities, depending on whether the acidic form, Eq. (*7*), or the basic one, Eq. (*8*), of the oxidant takes part in the reaction. The products formed in reactions represented by Eqs (*7*) and (*8*) are the reduced form of cytochrome c and the ascorbate free radical $A^{\bullet -}$.

Finally, $A^{\bullet -}$ reacts with either the acid or the basic form of the oxidant in Eqs (*9*) and (*10*), respectively, to give reduced cytochrome c and dehydroascorbic acid. Since the free radical is very unstable (and, so, very reactive), Eqs (*9*) and (*10*) are probably too fast to be rate determining steps.

According to the stoichiometry of the process, Eq. (*3*), the reaction rate can be defined as a half of the derivative of the concentration of rate-monitoring species (reduced cytochrome c) with respect to time:

$$r = (1/2)\,\mathrm{d}[\text{cyt–Fe}^{2+}]/\mathrm{d}t\,. \qquad (12)$$

Considering that reduced cytochrome c is formed in reactions represented by Eqs (*7*)–(*10*), we can write:

$$r = (1/2)\,(k_7[\text{AC–AH}^-] + k_8[\text{BC–AH}^-])\,[\text{OH}^-] + \\ + (1/2)\,(k_9[\text{AC}] + k_{10}[\text{BC}])\,[\text{A}^{\bullet -}]\,, \qquad (13)$$

where k_7, k_8, k_9 and k_{10} are the bimolecular rate constants corresponding to Eqs (*7*)–(*10*), whilst $AC–AH^-$ and $BC–AH^-$ stand for hydrogenascorbate ion attached to the acidic and basic forms of oxidized cytochrome c, respectively.

Now, application of the steady-state approximation to the free radical $A^{\bullet -}$ leads to:

$$(k_9[\text{AC}] + k_{10}[\text{BC}])\,[\text{A}^{\bullet -}] = \\ = (k_7[\text{AC–AH}^-] + k_8[\text{BC–AH}^-])\,[\text{OH}^-] \qquad (14)$$

and, from Eqs (*13*) and (*14*), we can infer:

$$r = (k_7[\text{AC–AH}^-] + k_8[\text{BC–AH}^-])\,[\text{OH}^-]\,. \qquad (15)$$

The following step in the development of the differential rate law based on the mechanism proposed (so that it can be tested with the experimental kinetic data) requires that the two alternatives for the interpretation of Eqs (*5*) and (*6*) be examined.

The Binding Hypothesis

Equations (*5*) and (*6*) can be interpreted as simple binding reactions, similar to the formation of the enzyme-substrate complex in the Michaelis–Menten mechanism for enzyme catalysis[16]. Let us assume as a reasonable approximation that the equilibrium constants for Eqs (*5*) and (*6*) are about the same, so that we can write:

$$[\text{AC–AH}^-]/[\text{AC}] = [\text{BC–AH}^-]/[\text{BC}] = K_b[\text{AH}^-]\,, \qquad (16)$$

where K_b is the equilibrium constant for the binding processes described by Eqs (*5*) and (*6*). In the mechanism proposed for the cytochrome c – ascorbic acid reaction, K_b plays the same role as the reciprocal of the Michaelis constant in the Michaelis–Menten mechanism for enzyme catalysis.

From Eqs (*15*) and (*16*) it follows that:

$$r = K_b[\text{AH}^-]\,[\text{OH}^-]\,(k_7[\text{AC}] + k_8[\text{BC}])\,. \qquad (17)$$

Considering that the total concentration of oxidized cytochrome c is:

$$[\text{CC}] = [\text{AC}] + [\text{BC}] + [\text{AC–AH}^-] + [\text{BC–AH}^-] \qquad (18)$$

from Eqs (*11*), (*16*) and (*18*) we can deduce:

$$[\text{AC}] = [\text{CC}]/x \qquad (19)$$

$$[\text{BC}] = K_4[\text{CC}]\,[\text{OH}^-]/x\,, \qquad (20)$$

where x stands for the expression:

$$x = (1 + K_b[\text{AH}^-])\,(1 + K_4[\text{OH}^-]) \qquad (21)$$

and by substitution of Eqs (*19*) and (*20*) in Eq. (*17*):

$$r = K_b[\text{CC}]\,[\text{AH}^-]\,[\text{OH}^-]\,(k_7 + K_4k_8[\text{OH}^-])/x\,. \qquad (22)$$

The concentration of hydroxyl ion can be written as a function of that of hydrogen ion

$$[OH^-] = K_w/[H^+], \tag{23}$$

where K_w is the water ionization constant. From Eqs (*21*)–(*23*)

$$r = K_w K_b[CC]\, yz, \tag{24}$$

where y and z are the function corresponding to the dependences of the reaction rate on the reductant and hydrogen ion concentrations, respectively

$$y = [AH^-]/(1 + K_b[AH^-]), \tag{25}$$

$$z = (k_7 + K_w K_4 k_8/[H^+])/(K_w K_4 + [H^+]). \tag{26}$$

Finally, since the reaction is first order in oxidized cytochrome c (Fig. 1 and Table I), the pseudo-first order rate constant can be obtained from the reaction rate as:

$$k_1 = r/[CC] \tag{27}$$

so that it follows from Eqs (*24*) and (*27*)

$$k_1 = K_w K_b yz. \tag{28}$$

The Adsorption Hypothesis

Equations (*5*) and (*6*) can be also interpreted as processes corresponding to the adsorption of hydrogenascorbate ion on the surface of the colloidal particles of oxidized cytochrome c. In a similar way as we did in relation with Eq. (*16*), let us assume as a reasonable approximation that the adsorption parameters are about the same for the acidic and basic forms of oxidized cytochrome c, so that we can write:

$$[AC\text{–}AH^-]/[AC] = [BC\text{–}AH^-]/[BC] = \\ = L_1[AH^-]/(1 + L_2[AH^-]), \tag{29}$$

where L_1 and L_2 are the Langmuir parameters[17] for the adsorption of hydrogenascorbate ion on the surface of oxidized cytochrome c (whether the acidic or the basic form).

From Eqs (*15*) and (*29*) it follows

$$r = L_1 y'[OH^-]\,(k_7[AC] + k_8[BC]), \tag{30}$$

where y' is the function corresponding to the dependence of the reaction rate on the reductant concentration

$$y' = [AH^-]/(1 + L_2[AH^-]) . \qquad (31)$$

The total concentration of oxidized cytochrome can be written now as

$$[CC] = [AC] + [BC] . \qquad (32)$$

It is important to notice the difference between Eqs (*18*) and (*32*). In the binding hypothesis it is necessary to consider the concentrations of all the forms of cytochrome c (acidic or basic, binded or free) when writing Eq. (*18*); however, in the adsorption hypothesis it is not possible to make a difference between free and non-free cytochrome c, since all the particles of cytochrome c are probably covered by adsorbed hydrogenascorbate ion, each particle with a certain degree of adsorption. Moreover, since each protein particle can be attached to several hydrogenascorbate ions, a balance of matter such as the one given in Eq. (*18*) would be now meaningless. Therefore, the concentrations of the acid and basic forms of cytochrome c in Eq. (*32*) have to be interpreted as total concentrations of those species, irrespective of whether hydrogenascorbate ion is adsorbed on them or not.

From Eqs (*11*) and (*32*) it can be inferred that:

$$[AC] = [CC]/(1 + K_4[OH^-]) , \qquad (33)$$

$$[BC] = K_4[CC]\,[OH^-]/(1 + K_4[OH^-]) \qquad (34)$$

and by substitution of Eqs (*23*), (*33*) and (*34*) in Eq. (*30*):

$$r = K_w L_1 [CC]\, y' z , \qquad (35)$$

where z has the same meaning as in Eq. (*26*).

Finally, from Eqs (*27*) and (*35*) we obtain:

$$k_1 = K_w L_1 y' z \qquad (36)$$

A Comparative Test of the Two Hypotheses

Both the binding and the adsorption hypotheses can explain most of the experimental results found in this work. Effectively, Eqs (*24*) and (*35*) are consistent with the first-order dependence on the oxidant concentration found for the reaction rate (see Fig. 1 and Table I).

As far as the reductant is concerned, both hypotheses lead to $1/k_1$ vs 1/[ascorbic acid] linear plots (see Fig. 2), what can be checked by examining Eqs (*25*), (*28*), (*31*) and (*36*). It should be remembered that, under the experimental conditions of this work, the concentration of hydrogenascorbate ion is equal to the total concentration of ascorbic acid for all practical purposes.

Both hypotheses can also explain the pH dependence found for the pseudo-first order rate constant, since Eqs (*26*), (*28*) and (*36*) lead to relationships between k_1 and $[H^+]$ of the kind experimentally found (see Fig. 4). According to those equations, parameter a appearing in Eq. (*2*) must be interpreted as:

$$a = K_w K_4 , \qquad (37)$$

whether the binding or the adsorption hypothesis is adopted. Considering that $K_w = 1{\cdot}01 \cdot 10^{-14}$ at 25·0°C (ref.[18]), from the experimental value found for parameter a (see Results) we can conclude that the constant for the acid-base equilibrium of oxidized cytochrome c, Eq. (*4*), is $K_4 = (6{\cdot}7 \pm 1{\cdot}4) \cdot 10^8$ at 25·0°C.

The effect of the ionic strength of the medium on the pseudo-first order rate constant is rather complex (see Table II), since it depends markedly on the temperature at which the reaction takes place. Whether the binding or the adsorption hypothesis holds, this effect is probably due to the interaction between hydrogenascorbate ion and the ionic double layer existing around each particle of cytochrome c.

We have seen that at high ionic strength the pseudo-first order rate constant fulfills the Arrhenius equation (Fig. 5), and that the resulting apparent activation energy increases with the pH of the solution (Table III). Again, both hypotheses can explain this behaviour, since from Eqs (*26*), (*28*) and (*36*) and the experimental values of the intercepts and slopes of the plots given in Fig. 4 it follows that k_7/k_8 decreases as the temperature increases (the approximate values of that ratio are 40 at 10·0°C and 20 at 25·0°C), and that the difference between the activation energies corresponding to rate constants k_8 and k_7 is:

$$E_{a,8} - E_{a,7} = 30 \pm 6 \text{ kJ mol}^{-1} . \qquad (38)$$

Hence, we can conclude that, under the experimental conditions of this work, the reduction of the acidic form of cytochrome c, Eq. (*7*), is faster than the reduction of the basic form, Eq. (*8*), although the difference decreases as the temperature increases due to the fact that the activation energy associated with the acidic form ($E_{a,7}$) is smaller than the activation energy associated with the basic form ($E_{a,8}$). This is precisely the reason why the apparent activation energy of the reaction increases with the pH of the solution (see Table III), because an increase in the pH results in an increase in the contribution of the basic form of oxidized cytochrome c

to the total reaction, and (as we have just seen) the basic form requires a higher activation energy for its reduction by hydrogenascorbate ion.

Actually, there are some reports[3,4] indicating that the basic form of oxidized cytochrome c might be irreducible by ascorbic acid, whilst the acidic form would be reducible. Our experimental results seem to indicate, however, that both forms of oxidized cytochrome c are reducible by ascorbic acid, but the rate constant for reduction of the acidic form is 20–40 times higher than the rate constant corresponding to the basic form. This difference in reactivity might indicate that the tertiary structure of the protein is involved in the reaction, and that a conformational change in that structure might result in important consequences as far as reducibility is concerned.

Although we can conclude that both hypotheses are consistent with the experimental kinetic data, only the binding hypothesis has received some attention in the literature published on the reaction[7,9,11]. However, the adsorption hypothesis might be somehow more attractive, because it is the only one that can properly explain the kind of behaviour found in Fig. 3. If we assume that, not only the Langmuir isotherm, but also the Freundlich isotherm is a good description for the adsorption processes given in Eqs (*5*) and (*6*), we can write instead of Eq. (*29*) this other:

$$[\mathrm{AC\text{–}AH^-}]/[\mathrm{AC}] = [\mathrm{BC\text{–}AH^-}]/[\mathrm{BC}] = F_1[\mathrm{AH^-}]^{F_2}\,, \qquad (39)$$

where F_1 and F_2 are the Freundlich parameters[19] for the adsorption of hydrogenascorbate ion on the surface of oxidized cytochrome c (whether the acidic or the basic form). Repeating the same development done for the case of the Langmuir isotherm (see above), we obtain now:

$$k_1 = K_w F_1 y'' z\,, \qquad (40)$$

where y'' is:

$$y'' = [\mathrm{AH^-}]^{F_2} \qquad (41)$$

and z has the same meaning as in Eq. (*26*). Hence, Eqs (*40*) and (*41*) can explain the log k_1 vs log [ascorbic acid] linear plots found in Fig. 3 (the total concentration of ascorbic acid is practically equal to the concentration of $\mathrm{AH^-}$). Moreover, according to the Freundlich isotherm, F_2 must be a number between 0 and 1 (ref.[19]), and this is in perfect agreement with the experimental values found for the plots given in Fig. 3 ($F_2 = 0{\cdot}42 \pm 0{\cdot}03$, $0{\cdot}53 \pm 0{\cdot}01$ and $0{\cdot}53 \pm 0{\cdot}02$ at 10·0, 15·0 and 20·0°C, respectively).

Thus, we can see that, although there is not a definitive proof supporting one of the two possible hypotheses, adsorption might be a better explanation than binding. The difficulty of making a definitive choice rises from the close similitude between

the two hypotheses, since both require the linkage of the reductant to the oxidant. Actually, the two hypotheses differ only in the number of active sites on the protein surface for the linkage of the reductant; a single active site would mean binding, whereas several active sites would mean adsorption. We believe that more experimental information is necessary in order to make a definitive choice between the two possible explanations for the reaction.

REFERENCES

1. Hodges H. L., Holwerda R. A., Gray H. B.: J. Am. Chem. Soc. *96*, 3132 (1974).
2. Jaffe G. M. in: *Encyclopedia of Chemical Technology*, 3rd ed. (M. Grayson, Ed.), Vol. 24, p. 8. Wiley, New York 1984.
3. Greenwood C., Palmer G.: J. Biol. Chem. *240*, 3660 (1965).
4. Wilson M. T., Greenwood C.: Eur. J. Biochem. *22*, 11 (1971).
5. Al-Ayash A. I., Wilson M. T.: Biochem. J. *177*, 641 (1979).
6. Goldkorn T., Schejter A.: J. Biol. Chem. *254*, 12562 (1979).
7. Myer Y. P., Thallam K. K., Pande A.: J. Biol. Chem. *255*, 9666 (1980).
8. Heremans K., Bormans M., Snauwaert J., Vandersypen H.: Faraday Discuss. Chem. Soc. *74*, 343 (1982).
9. Myer Y. P., Kumar S.: J. Biol. Chem. *259*, 8144 (1984).
10. Williams N. H., Yandell J. K.: Biochim. Biophys. Acta *810*, 274 (1985).
11. Mathews A. J., Brittain T.: Biochem. J. *243*, 379 (1987).
12. Theorell H., Akesson A.: Science *90*, 67 (1939).
13. Greenwood C., Wilson M. T.: Eur. J. Biochem. *22*, 5 (1971).
14. Macartney D. H., McAuley A.: Can. J. Chem. *59*, 132 (1981).
15. Kimura M., Yamamoto M., Yamabe S.: J. Chem. Soc., Dalton Trans. *1982*, 423.
16. Laidler K. J., Bunting P. S.: *The Chemical Kinetics of Enzyme Action*, 2nd ed., p. 72. Clarendon, Oxford 1973.
17. Langmuir D. in: *Adsorption from Aqueous Solutions* (P. H. Tewari, Ed.), p. 2. Plenum, New York 1981.
18. Coetzee J. F., Ritchie C. D.: *Solute–Solvent Interactions*, p. 5. Dekker, New York 1969.
19. Parfitt G. D., Rochester C. H. in: *Adsorption from Solution at the Solid/Liquid Interface* (G. D. Parfitt and C. H. Rochester, Eds), p. 3. Academic Press, New York 1983.