KINETICS AND MECHANISM OF THE CYTOCHROME c – – SULFITE REACTION

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The reaction between the oxidized form of horse-heart cytochrome c and sodium sulfite in aqueous solution has been studied in the pH range 6·5—8·2. The reaction is first order in both oxidant and reductant, is accelerated by an increase in pH and is slowed down by addition of potassium chloride. An increase in pH results in an increase in the apparent activation energy (66—77 kJ . . mol^{-1}). A mechanism in which both HSO_3^- and SO_3^{2-} act as reducing agents is proposed, the activation energies corresponding to the cyt c–HSO_3^- and cyt c–SO_3^{2-} reactions being 63 ± 4 and 79 ± 2 kJ mol^{-1}, respectively.

The oxidized and reduced forms of cytochrome c play an important role in the mitochondrial respiratory chain[1]. Thus the redox properties of cytochrome c have received a considerable attention, both from the perspectives of electrochemistry[2,3] and chemical kinetics[4]. Although many kinetic studies on the reduction of oxidized cytochrome c by some physiological (such as ascorbate ion[5,6]) and nonphysiological (such as dithionite ion[6,7]) reducing agents have been reported, the reduction of oxidized cytochrome c by sulfite ion (although it is known to occur[8]) has not received a similar attention.

Now we report a kinetic study on the reaction:

$$2\ \text{cyt-Fe}^{3+} + SO_3^{2-} + H_2O \rightarrow 2\ \text{cyt-Fe}^{2+} + SO_4^{2-} + 2\ H^+ \qquad (1)$$

(where cyt-Fe^{3+} and cyt-Fe^{2+} are the oxidized and reduced forms of cytochrome c, respectively) in aqueous solutions near the neutrality region (pH 6·5—8·2). Both HSO_3^- and SO_3^{2-} have been found to reduce oxidized cytochrome c in that pH region, and the activation parameters corresponding to both the cyt c–HSO_3^- and cyt c–SO_3^{2-} reactions have been determined.

EXPERIMENTAL

The oxidant was horse-heart cytochrome c in its oxidized form (Sigma, Type III). The reductant was sodium sulfite (Merck, analytical grade). All the other chemical compounds used (KH_2PO_4,

K_2HPO_4, KCl and Na_2SO_4) were also of analytical grade (Merck). The solvent was twice-distilled water. The cytochrome c stock solutions were prepared just prior to the experiments and the sodium sulfite stock solutions were prepared daily (in water previously deoxygenated by bubbling of argon).

The reductant (Na_2SO_3) was always in large excess with respect to oxidant (cytochrome c). The pH of the solutions was kept constant during the kinetic runs with the use of KH_2PO_4–K_2HPO_4 buffers (pH 6·5—8·2). The ionic strength was kept constant with the use of either Na_2SO_4 (in the experiences where the Na_2SO_3 concentration was changed) or KCl (in the experiences where the concentrations of KH_2PO_4 and K_2HPO_4 were changed).

The reaction was monitored by following the formation of reduced cytochrome c at 550 nm in a Varian Cary 219 UV-vis spectrophotometer (glass cuvettes, pathlength 1 cm). The pH values were measured with a Metrohm 605 pH-meter provided with a glass–calomel combination electrode. The temperature was kept constant during the kinetic runs (range 14·8—35·2°C) with the aid of a conventional thermostat. Since some manipulations (mixing of reactants and filling of the cuvettes) had to be necessarily done at room temperature, the temperature of the laboratory was kept as close as possible to that of the thermostat (average difference 0·8°C). This precaution was especially important in the determination of the activation parameters.

RESULTS

Although the initial rate was directly proportional to the initial cytochrome c concentration (Table I), thus indicating that the reaction was of first order with respect to oxidant, the $\ln(A_\infty - A)$ vs time plots presented in all of the cases a definite concave-upward curvature (see Fig. 1 for a typical example). Because of this, the kinetic parameters were obtained by means of the initial-rate method, the first-order rate constant being calculated as the ratio between the initial rate and the initial cytochrome c concentration ($k_1 = r_0/[\text{cyt c}]_0$). For each k_1 value 2—4 independent determinations were made, and the average standard deviation for the k_1 values was 3·3% (100 experiences). The first-order rate constant was directly proportional

TABLE I

Initial rates and first-order rate constants at various cytochrome c initial concentrations ($[Na_2SO_3] = 0{\cdot}20$ mol dm^{-3}, pH 7·25, ionic strength 1·08 mol dm^{-3}, 25·0°C)

$[\text{cyt c}]_0 \cdot 10^5$ mol dm^{-3}	$r_0 \cdot 10^8$ mol dm^{-3} s^{-1}	$k_1 \cdot 10^{3a}$ s^{-1}
0·95	1·80 ± 0·03	1·89 ± 0·03
1·27	2·40 ± 0·07	1·89 ± 0·05
1·59	3·05 ± 0·09	1·92 ± 0·06
1·91	3·62 ± 0·03	1·90 ± 0·02
2·22	4·24 ± 0·16	1·91 ± 0·07

[a] Obtained as $k_1 = r_0/[\text{cyt c}]_0$.

to the sulfite concentration (Fig. 2), thus indicating that the kinetic order of the reductant was also unity.

The reaction was accelerated by an increase in the alkalinity of the solution (pH range 6·5–8·2). Actually, k_1 was found to follow the equation:

$$(1 + a[H^+])/k_1 = b + c[H^+], \qquad (2)$$

where parameter a was optimized so that the $(1 + a[H^+])/k_1$ vs $[H^+]$ plots were linear, whereas parameters b and c were obtained from the intercepts and slopes of those plots, respectively. Equation (*2*) can also be written as:

$$\log [(1 + a[H^+])/k_1 - b] = \log c - \mathrm{pH}\,. \qquad (3)$$

The $\log [(1 + a[H^+])/k_1 - b]$ vs pH plots (slope = −1) were obtained at five different temperatures in the range 14·8–35·2°C (see Fig. 3 for a typical example).

The reaction was slowed down by addition of potassium chloride to the solution (Table II). Finally, the first-order rate constant followed the Arrhenius equation at the five pH values studied (Fig. 4), and the corresponding apparent activation energy showed a definite increase with increasing pH (Table III).

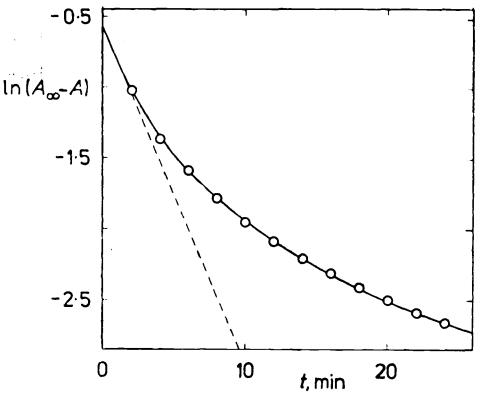

FIG. 1

Attempted first-order plot. [cyt c] = 2·20 . 10^{-5} mol dm^{-3}; $[Na_2SO_3]$ = 0·20 mol . dm^{-3}; pH 7·25; ionic strength 1·08 mol . dm^{-3}; 25·0°C. The dashed line represents the tangent to the curve at the beginning of the reaction

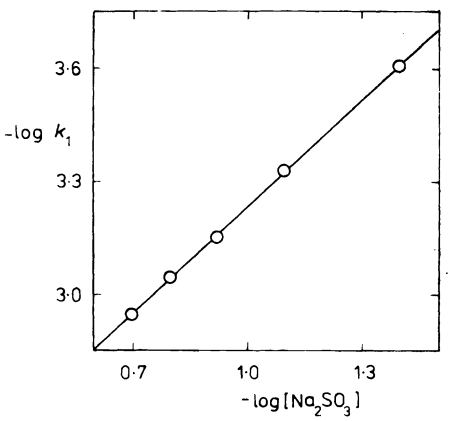

FIG. 2

Dependence of the first-order rate constant on the sodium sulfite concentration. [cyt c] = = 1·60 . 10^{-5} mol dm^{-3}; pH 6·78; ionic strength 1·58 mol dm^{-3} (Na_2SO_4); 25·0°C. Slope 0·94 ± 0·01

TABLE II

First-order rate constants at various potassium chloride concentrations ([cyt c] = 1·60 . 10^{-5} mol dm^{-3}, [Na_2SO_3] = 0·20 mol dm^{-3}, pH 7·24, ionic strength 1·08 + [KCl] mol . dm^{-3}, 25·0°C)

[KCl] mol dm^{-3}	$k_1 . 10^3$ s^{-1}
0·00	1·87 ± 0·06
0·24	1·56 ± 0·01
0·48	1·38 ± 0·02
0·72	1·19 ± 0·05
0·96	1·09 ± 0·12

TABLE III

Apparent activation energies at various pH values. For the experimental conditions see Fig. 4

pH	6·47	6·77	7·10	7·51	8·22
E_a, kJ mol^{-1}	66 ± 4	68 ± 3	69 ± 4	76 ± 2	77 ± 2

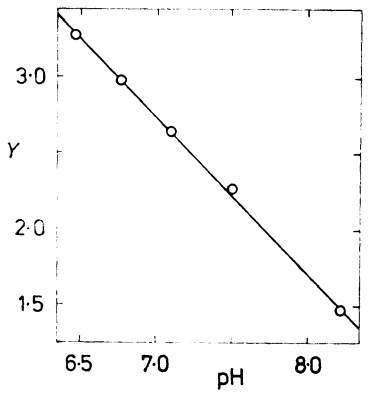

FIG. 3

Dependence of the first-order rate constant on the pH. [cyt c] = 1·60 . 10^{-5} mol dm^{-3}; [Na_2SO_3] = 0·20 mol dm^{-3}; ionic strength 1·27 mol dm^{-3} (KCl); 25·0°C. *Y* stands for log [(1 + $a[H^+]$)/k_1 − *b*]. Slope −1·03 ± 0·02

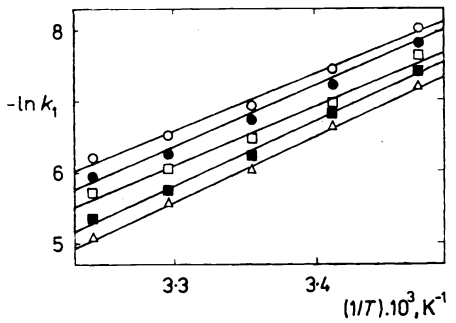

FIG. 4

Arrhenius plots for the first-order rate constant at various pH values. [cyt c] = = 1·60 . 10^{-5} mol dm^{-3}; [Na_2SO_3] = = 0·20 mol dm^{-3}; pH 6·47 (○), 6·77 (●), 7·10 (□), 7·51 (■) and 8·22 (△); ionic strength 1·27 mol dm^{-3} (KCl)

DISCUSSION

In the near-neutrality pH region studied in this work (pH 6·5–8·2) the reductant was present predominantly as hydrogensulfite and sulfite ions, according to the equilibrium

$$HSO_3^- \rightleftarrows SO_3^{2-} + H^+ . \qquad (4)$$

Hence, both HSO_3^- and SO_3^{2-} can act as reducing agents for the oxidized form of cytochrome c. The mechanism that can be proposed for the reaction is

$$\text{cyt-Fe}^{3+} + HSO_3^- \xrightarrow{\text{slow}} \text{cyt-Fe}^{2+} + HSO_3^{\bullet} \qquad (5)$$

$$\text{cyt-Fe}^{3+} + HSO_3^{\bullet} + H_2O \xrightarrow{\text{fast}} \text{cyt-Fe}^{2+} + SO_4^{2-} + 3\,H^+ \qquad (6)$$

$$\text{cyt-Fe}^{3+} + SO_3^{2-} \xrightarrow{\text{slow}} \text{cyt-Fe}^{2+} + SO_3^{\bullet -} \qquad (7)$$

$$\text{cyt-Fe}^{3+} + SO_3^{\bullet -} + H_2O \xrightarrow{\text{fast}} \text{cyt-Fe}^{2+} + SO_4^{2-} + 2\,H^+ . \qquad (8)$$

It should be noticed that, although the steps corresponding to Eqs (*6*) and (*8*) imply trimolecular encounters, one of the molecules belongs to the solvent (in large excess with respect to the other reactants); therefore, those reactions are also elementary.

The rate of reaction will be given by the disappearance of oxidized cytochrome c through the slow steps

$$r = (k_5[HSO_3^-] + k_7[SO_3^{2-}])\,[\text{cyt-Fe}^{3+}] , \qquad (9)$$

where k_5 and k_7 are the second-order rate constants corresponding to Eqs (*5*) and (*7*), respectively. Considering that HSO_3^- and SO_3^{2-} are in equilibrium, we obtain for the reaction rate

$$r = (k_5[H^+] + K_4k_7)\,[\text{cyt-Fe}^{3+}]\,[Na_2SO_3]_T/(K_4 + [H^+]) \qquad (10)$$

and for the first-order rate constant

$$k_1 = r/[\text{cyt-Fe}^{3+}] = (k_5[H^+] + K_4k_7)\,[Na_2SO_3]_T/(K_4 + [H^+]) , \qquad (11)$$

where K_4 and $[Na_2SO_3]_T$ are the equilibrium constant corresponding to Eq. (*4*) and the total sodium sulfite concentration, respectively.

Equations (*10*) and (*11*) are in agreement with the experimental results found in this work. In the first place, according to Eq. (*10*) the kinetic order of oxidized cytochrome c must be unity, what is in agreement with the results given in Table I.

Although the first-order plots were not linear, $\ln(A_\infty - A)$ vs time plots similar to that shown in Fig. 1 have been reported for the reactions of oxidized cytochrome c with other reductants such as ascorbate ion[9-11]. This behaviour is probably due to the existence of two or more conformers of oxidized cytochrome c with different degrees of reducibility. As the reaction advances, the proportion of the less-reducible conformers of oxidized cytochrome c increases, what results in the $\ln(A_\infty - A)$ vs time plots showing a concave-upward curvature (the $r/[\text{cyt c}]$ ratio decreases as time increases). Since in this work the initial-rate method has been used, the results found concern the most reducible conformer of oxidized cytochrome c (provided that the difference between its reducibility and that of the other conformers be big enough, what agrees with previous assumptions concerning those conformers[9]).

Equations (*10*) and (*11*) are also in agreement with the kinetic order unity found for the reductant (Fig. 2). It is important to notice that the fact that the rate is directly proportional to the reductant concentration makes unneccessary the proposal of a binding step between oxidant and reductant previous to the redox reaction, what contrasts with the results found for the cytochrome c–ascorbate reaction[6,10,11].

Moreover, Eq. (*11*) is also in agreement with the pH-dependence found for k_1 (Fig. 3). Effectively, a comparison of Eqs (*2*) and (*11*) leads to the following identities:

$$a = k_5/K_4k_7 \qquad (12)$$

$$b = 1/k_7[\text{Na}_2\text{SO}_3]_\text{T} \qquad (13)$$

$$c = 1/K_4k_7[\text{Na}_2\text{SO}_3]_\text{T} \,. \qquad (14)$$

Therefore, from Eqs (*12*)–(*14*) and the experimental data obtained in this work it has been possible to determine the values of the second-order rate constants for the cyt c–HSO_3^- and cyt c–SO_3^{2-} reactions (k_5 and k_7, respectively), as well as that of the second dissociation constant of sulfurous acid (K_4). The average value obtained in this work for the later is $pK_4 = 7{\cdot}13 \pm 0{\cdot}12$, in good agreement with the value reported in the literature[12] ($pK_4 = 6{\cdot}91$).

It has to be noticed that the pH-dependence found for k_1 has been related to the existence of several acid-base forms of the reductant, and not to the existence of several acid-base forms of the oxidant. This is indeed a correct assumption for, although it is known that oxidized cytochrome c has four different acid-base forms in pH-related equilibrium, the corresponding pK_a values (2·5, 9·5 and 12·7 (ref.[13])) are all outside of the pH range studied in this work (6·5–8·2), whereas the second pK_a of sulfurous acid (6·91 (ref.[12])) is within that pH range. This means that in the pH range studied, the reductant was present mainly in two acid-base forms (HSO_3^- and SO_3^{2-}), whereas the oxidant was present predominantly in a single acid-base form (the second more protonated one, whose pK_a is 9·5).

The decrease found in k_1 with increasing concentration of potassium chloride (Table II) can be related to the formation of an electric double layer[14] around the protein molecules, as it is known to occur with macromolecules and colloidal particles in aqueous solution. An increase in the potassium chloride concentration results in an increase in the thickness of that electric double layer, what makes more difficult the approach of HSO_3^- or SO_3^{2-} to the heme prosthetic group of oxidized cytochrome c where the iron(III) atom is located, thus resulting in a decrease in k_1.

Both second-order rate constants k_5 and k_7, obtained from Eqs (*12*)–(*14*), fulfill the Arrhenius equation (Fig. 5). The corresponding activation parameters (that is, the ones associated with the cyt c–HSO_3^- and cyt c–SO_3^{2-} reactions) are given in Table IV. We can see that the activation energy associated with k_5 (63 ± 4 kJ . . mol^{-1}) is lower than the one associated with k_7 (79 ± 2 kJ mol^{-1}). This can indeed explain the increase found in the apparent activation energy (associated with k_1) with increasing alkalinity of the solution (Table III), for an increase in the pH results in an increase in the contribution of the cyt c–SO_3^{2-} reaction (higher activat-

TABLE IV

Activation parameters corresponding to the cyt c–HSO_3^- and cyt c–SO_3^{2-} reactions. For the experimental conditions see Fig. 5

Reductant	E_a kJ mol^{-1}	$\Delta H^{\neq}$ kJ mol^{-1}	$-\Delta S^{\neq a}$ J K^{-1} mol^{-1}
HSO_3^-	63 ± 4	61 ± 4	89 ± 12
SO_3^{2-}	79 ± 2	77 ± 2	25 ± 6

[a] The activation entropies are referred to the 1 mol dm^{-3} standard state.

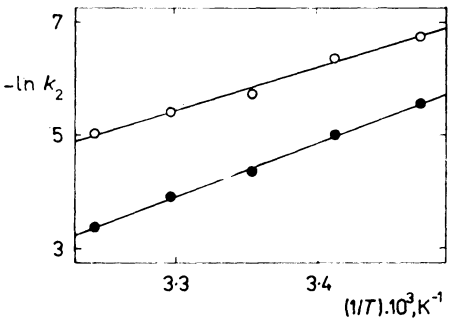

FIG. 5

Arrhenius plots for the second-order rate constants corresponding to the cyt c–HSO_3^- (○) and cyt c–SO_3^{2-} (●) reactions. [cyt c] = = $1{\cdot}60 \cdot 10^{-5}$ mol dm^{-3}; $[Na_2SO_3]$ = = 0·20 mol dm^{-3}; pH 6·47–8·22; ionic strength 1·27 mol dm^{-3} (KCl)

ion energy) and a decrease in the contribution of the cyt c–HSO_3^- one (lower activation energy). Finally, it should be mentioned that the k_7/k_5 ratio was bigger than unity at all the five temperatures studied ($k_7/k_5 = 3{\cdot}3-5{\cdot}2$ in the range 14·8 to 35·2°C). This indicates that SO_3^{2-} is a better reductant for the oxidized form of cytochrome c than HSO_3^-, probably due to the higher electron density of the fully-deprotonated ion.

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