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THE OXIDATION-REDUCTION KINETICS OF THE REACTION OF CYTOCHROME c_1 WITH NON-PHYSIOLOGICAL REDOX AGENTS

B.W. KÖNIG, E.C.I. VEERMAN and B.F. VAN GELDER

Laboratory of Biochemistry, B.C.P. Jansen Institute, University of Amsterdam, P.O. Box 20151, 1000 HD Amsterdam (The Netherlands)

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The kinetics of the oxidation-reduction reactions of cytochrome c_1 with ascorbate, ferricyanide, triphenanthrolinecobalt(III) and *N,N,N′,N′*-tetramethyl-*p*-phenylenediamine (TMPD) have been examined using the stopped-flow technique. The reduction of ferricytochrome c_1 by ascorbic acid is investigated as a function of pH. It is shown that at neutral and alkaline pH the reduction of the protein is mainly performed by the doubly deprotonated form of ascorbate. From the ionic-strength-dependence studies of the reactions of cytochrome c_1 with ascorbate, ferricyanide and triphenanthrolinecobalt(III), it is demonstrated that the reaction rate is governed by electrostatic interactions. The second-order rate constants for the reaction of cytochrome c_1 with ascorbate, ferricyanide, TMPD and triphenanthrolinecobalt(III) are $1.4 \cdot 10^4$, $3.2 \cdot 10^3$, $3.8 \cdot 10^4$ and $1.3 \cdot 10^8$ $M^{-1} \cdot s^{-1}$ (pH 7.0, $I = 0$, 10°C), respectively. Application of the Debye-Hückel theory to the data of the ionic-strength-dependence studies of these redox reactions of cytochrome c_1 yielded for ferrocytochrome c_1 and ferricytochrome c_1 a net charge of -5 and -4, respectively. The latter value is close to that of -3 for the oxidized enzyme, calculated from the amino acid sequence of the protein. This implies that not a local charge on the surface of the protein, but the overall net charge of cytochrome c_1 governs the reaction rate with small redox molecules.

Introduction

Cytochrome c_1, one of the constituents of the mitochondrial ubiquinol : ferricytochrome *c* oxidoreductase complex (Complex III), is involved in the electron transfer from the Rieske iron-sulphur protein to cytochrome *c*. The isolation of purified cytochrome c_1 on a large scale [1] has enabled us to characterize this protein by studying its physico-chemical properties.

In previous papers, we investigated the kinetics of the reaction of cytochrome c_1 with various protein redox partners, such as cytochrome *c*, cytochrome aa_3 and the cytochrome *c*-aa_3 complex [2,3]. As a result of these studies, it was demonstrated that the rate of the reaction between cytochrome c_1 and cytochrome *c* is fast ($k_1 = 3 \cdot 10^7$ $M^{-1} \cdot s^{-1}$, pH 7.0, $I = 0.2$ M, 10°C) and that electrostatic interactions govern the reaction rate [2]. Furthermore, it was shown by Wilms et al. [3] that the reduction of the cytochrome *c*-aa_3 complex by ferrocytochrome c_1 does not occur, but proceeds via non-complex-bound cytochrome *c*.

Additional information concerning the physico-chemical properties of cytochrome c_1 has been obtained from kinetic studies of the reaction between cytochrome c_1 and singly substituted cytochrome *c* derivatives [4]. It was demonstrated that lysine residues 13, 27, 72, 86 and 87 of cytochrome *c* are involved in the interaction with

Abbreviations: TMPD, *N,N,N′,N′*-tetramethyl-*p*-phenylenediamine; Hipip, high-potential Fe-S protein; CDNP, 4-carboxy-2,6-dinitrophenyl.

cytochrome c_1. The interaction domain on cytochrome c for cytochrome c_1, defined in this way, was found to be identical to that determined for cytochrome aa_3 [5].

In the present paper, we have further characterized cytochrome c_1 by studying in more detail the reaction of cytochrome c_1 with several non-physiological redox agents. These studies provide information about the electron-transfer reactions in the terminal part of the mitochondrial respiratory chain. Furthermore, from the ionic-strength dependence of the reactions of cytochrome c_1 with small ions, we estimated the effective net charge of cytochrome c_1 involved in the electron-transfer reaction (cf. Ref. 6).

Materials and Methods

Reduced bovine cytochrome c_1 was isolated and purified as described before [1]. In order to remove endogenous reducing equivalents in the cytochrome c_1 preparation [7], the protein was treated with an excess of sodium dithionite and eluted on Sephadex G-50 superfine in 5 mM potassium phosphate (pH 7.0), 1% Tween 20 at 4°C. Ferricytochrome c was prepared by oxidation of ferrocytochrome c_1 with a minimal amount of $K_3Fe(CN)_6$ and subsequently the mixture was passed through a column (25 × 0.5 cm) of Sephadex G-50 superfine in 5 mM potassium phosphate (pH 7.0), 1% Tween 20 at 4°C. Immediately before use the oxidized or reduced protein was desalted on a column (25 × 0.5 cm) of Sephadex G-50 superfine in 2 mM potassium phosphate buffer (pH 7.0) and 1% Tween 20 at 4°C.

Ascorbic acid (Merck) was prepared freshly and brought to pH 7.0 with KOH; in order to prevent oxidation of ascorbic acid by oxygen, the solutions of the salt were saturated with nitrogen for 30 min. Stock solutions of $K_3Fe(CN)_6$ and $Co(phen)_3(ClO_4)_3 \cdot 2H_2O$ (a gift from Dr. Sykes of the University of Newcastle, U.K.) were prepared freshly in H_2O. Tween 20 was obtained from Serva.

The concentrations of cytochrome c_1 and triphenanthrolinecobalt(III) ($Co(phen)_3^{3+}$) were determined spectrophotometrically using absorbance coefficients for the protein of 19.2 $mM^{-1} \cdot cm^{-1}$ (reduced minus oxidized) at 552 nm [7,8] and for the oxidizing reagent of $4.68^{-1} \cdot cm^{-1}$ at 330 nm [9].

The ionic strength was changed either by addition of KCl or by increasing the concentration of potassium phosphate buffer. No specific anion effect of chloride in comparison to phosphate was observed on the oxidation-reduction kinetics of cytochrome c_1.

The redox reactions of cytochrome c_1 were studied using a Durrum-Gibson stopped-flow apparatus by monitoring the change in absorbance at 417 or 552.5 nm at 10°C. Storage and processing of the absorbance traces were carried out as described before [10]. At low ionic strength ($I < 30$ mM), the reaction of cytochrome c_1 with ascorbate or $K_3Fe(CN)_6$ was studied under conditions in which the ratio of concentrations of reductant to protein and of oxidant to protein were 1000 and 250, respectively. For the oxidation of ferrocytochrome c_1 by $Co(phen)_3^{3+}$ a 10-fold molar excess of this reagent was used, since at low ionic strength ($I < 30$ mM) higher concentrations of $Co(phen)_3^{3+}$ resulted in completion of the reaction within the mixing time (3 ms) of our stopped-flow apparatus. Under conditions of high ionic strength ($I > 100$ mM), the oxidation of ferrocytochrome c_1 by $K_3Fe(CN)_6$ showed monophasic kinetics, whereas at low ionic strength ($I < 30$ mM) biphasic behaviour was observed. The first phase of the latter reaction could be fitted by one exponential and contributed to at least 75% of the total absorbance change. The observed rate constant (k') for this phase increased linearly with the concentration of $K_3Fe(CN)_6$, whereas the second phase was independent of the concentration of oxidant.

The data of the experiments concerning the ionic-strength dependence of the redox reactions of cytochrome c_1 were analyzed using the Brønsted-Debye-Hückel equation [11], which describes the relationship between the observed rate constant (k) and the ionic strength (I):

$$\log k_I = \log k_{I=0} - \frac{Z_A^2 \alpha \sqrt{I}}{1 + \beta \cdot R_A \sqrt{I}} - \frac{Z_B^2 \alpha \sqrt{I}}{1 + \beta \cdot R_B \sqrt{I}} + \frac{(Z_A + Z_B)^2 \alpha \sqrt{I}}{1 + \beta \cdot R_{AB} \sqrt{I}} \quad (1)$$

where α and β are constants with values of 0.499

and 3.27 $nm^{-1} \cdot M^{-1/2}$, respectively, at 10°C; k_I is the rate constant at a particular ionic strength; Z_A and Z_B are the charges of the reactants A and B and R_A, R_B and R_{AB^*} are the radii of the reactants and the transition complex, respectively. By introducing into Eqn. 1, $R_A = R_{AB^*}$, i.e., if $R_A \gg R_B$, we obtained the frequently used form of the Brønsted-Debye-Hückel equation for the ionic-strength dependence of the reaction between a large molecule, such as a protein, and a small ion [11]:

$$\log k_I = \log k_{I=0} - \frac{Z_B^2 \alpha \sqrt{I}}{1+\beta R_B \sqrt{I}} + \frac{(2Z_A Z_B + Z_B^2)\,\alpha\sqrt{I}}{1+\beta R_A \sqrt{I}} \quad (2)$$

The radii of ascorbate, $Fe(CN)_6^{3-}$ and $Co(phen)_3^{3+}$ were taken as 0.40, 0.45 and 0.70 nm, respectively [6], whereas the radius of cytochrome c_1 was calculated using the equation $R(nm) = 0.0717 M_r^{1/3}$ [6]. For cytochrome c_1 ($M_r = 31000$ [1]) a value of 2.25 nm was obtained. Eqn. 2 was used in the analysis of the data of the experiments described in this paper.

Results

Fig. 1A shows the relationship between the pH and the second-order rate constant (k_{app}). In the pH range studied, the reduction of ferricytochrome c_1 by ascorbate showed monophasic behaviour and under pseudo-first-order conditions (ascorbate in excess with respect to cytochrome c_1) the observed rate constant was found to be linearly dependent on the concentration of ascorbate. From Fig. 1A it can be seen that above pH 7.0, the rate of the reaction increases rapidly. A plot of the apparent second-order rate constant ($\log k_{app}$) vs. pH (Fig. 1B) yields a straight line with a slope of unity in the pH range 7–10, whereas deviation from linearity is observed below neutral pH.

If both the singly (AH^-) and doubly (A^{2-}) deprotonated forms of ascorbate reduce cytochrome c_1, the rate of reduction is:

$$\frac{d[\text{cytochrome } c_1^{2+}]}{dt} = \{k_1[AH^-] + k_2[A^{2-}]\}[\text{cytochrome } c_1^{3+}] \quad (3)$$

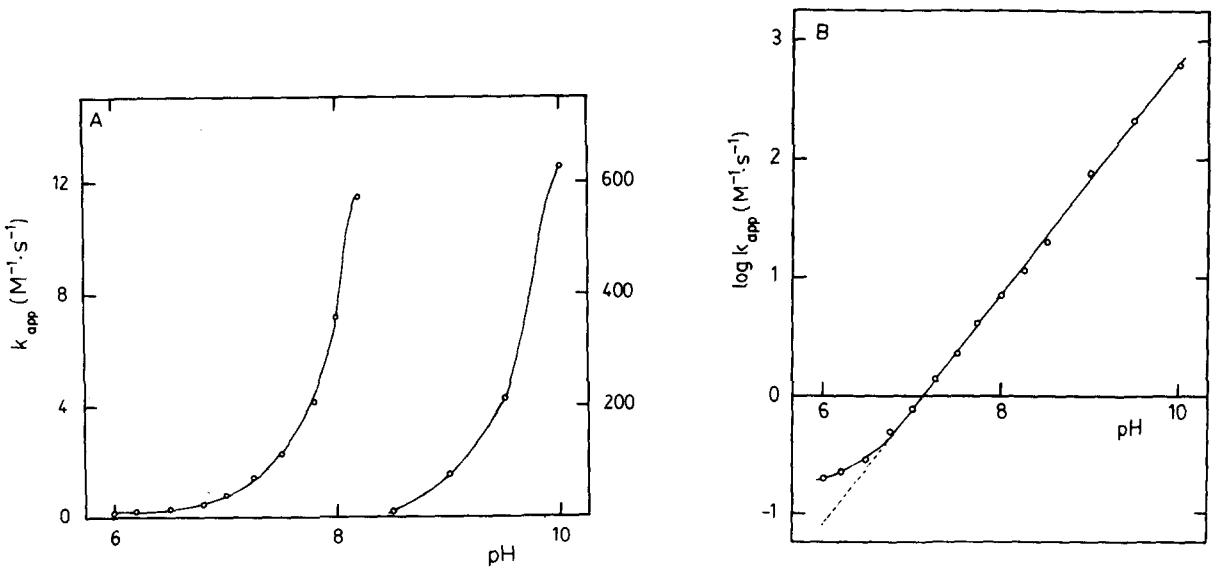

Fig. 1 (A) Effect of the pH on the apparent second-order rate constant of the reaction between ferricytochrome c_1 and ascorbate. Conditions: 50 mM potassium phosphate (pH 7.0), 1% Tween 20. Variation in the ionic strength as a consequence of changing the pH was compensated by the addition of KCl, until an ionic strength of 0.7 M was obtained. Final concentrations of ascorbate and ferricytochrome c_1 were 4 mM and 1.95 μM, respectively. The values of the apparent second-order rate constant in the pH range 6–8.2 are given with reference to the left-hand ordinate and in the pH range 8.5–10 with reference to the right-hand ordinate. (B) Relationship between the logarithm of the apparent second-order rate constant of the reaction between ferricytochrome c_1 and ascorbate, vs. pH. Conditions as in A.

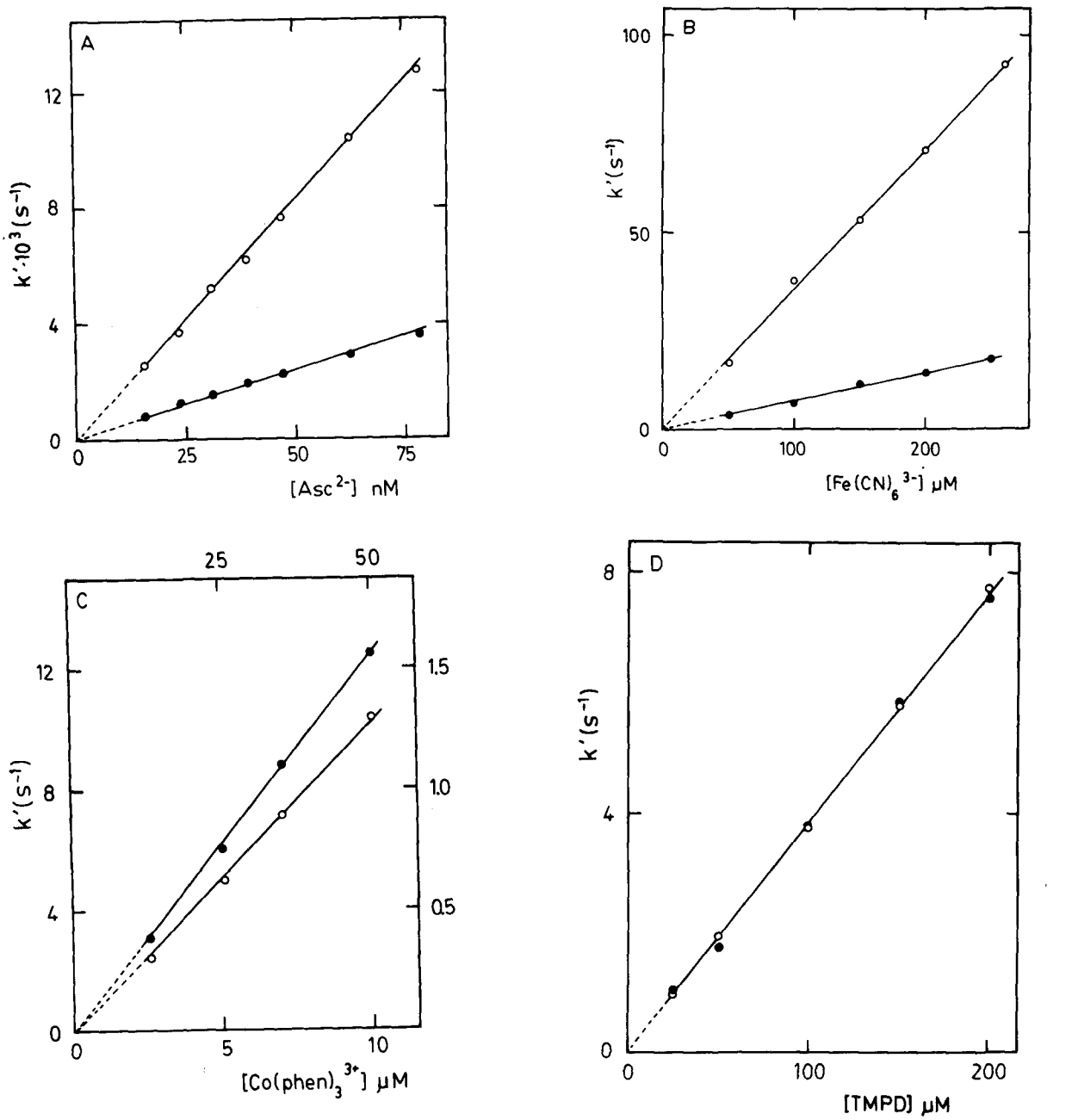

Fig. 2. Pseudo-first order rate constant of the reaction between cytochrome c_1 and non-physiological redox agents, as a function of the concentration of the agents. The reactions were carried out in 50 mM potassium phosphate (pH 7.0), 1% Tween 20 (●) and KCl ($I = 1.1$ M) (○). (A) The reaction of ferricytochrome c_1 (1.95 μM) with ascorbate. The potassium phosphate concentration was 5 mM instead of 50 mM. The effect of the addition of increasing amounts of ascorbate on the ionic strength was compensated by KCl, until an ionic strength of 30 mM was obtained (○). (B) The reaction of ferrocytochrome c_1 (0.9 μM) with $K_3Fe(CN)_6$. (C) The reaction of ferrocytochrome c_1 (0.9 μM) with $Co(phen)_3^{3+}$. The values of the rate constant and the concentration of $Co(phen)_3^{3+}$ at lower ionic strength are plotted with regard to the left-hand and bottom axes, the values at higher ionic strength with regard to the right-hand and top axes. (D) The reaction of ferricytochrome c_1 (0.9 μM) with TMPD. The TMPD was kept fully reduced by the addition of a 4-fold molar excess of ascorbate.

in which k_1 and k_2 are the second-order rate constants for the reaction of cytochrome c_1 with the singly and doubly negatively charged ascorbate ion, respectively. As the dissociation constants of ascorbic acid are $5.1 \cdot 10^{-5}$ M (K_1) and $7.8 \cdot 10^{-13}$ M (K_2), interpolated from values determined at 0.4 and 25°C [12], the main species present in the pH range of these experiments is the singly deprotonated form of ascorbate. From Eqn. 3 and the Henderson-Hasselbalch equation it is derived that:

$$k_{app} = k_1 + k_2 \cdot \frac{K_2}{[H^+]} \quad (4)$$

As $\log k_{app}$ is a linear function of pH (pH 7–10) (Fig. 1B), the value of k_1 in this pH range must be small with respect to k_{app}. Therefore, at neutral and alkaline pH cytochrome c_1 is mainly reduced by the doubly deprotonated form of ascorbate, whereas at acidic pH the reduction of cytochrome c_1 by the singly negatively charged ascorbate ion prevails.

Fig. 2 shows that the pseudo-first-order rate constant (k') for the reaction of cytochrome c_1 with ascorbate^{2-}, $Fe(CN)_6^{3-}$, $Co(phen)_3^{3+}$ and TMPD is linearly dependent on the concentration of oxidant or reductant at different ionic strengths.

TABLE I

IONIC-STRENGTH STUDIES OF THE REACTION OF CYTOCHROME c_1 WITH NON-PHYSIOLOGICAL REDOX AGENTS

Conditions: potassium phosphate (pH 7.0), 1% Tween 20 and KCl

Reaction	Ionic strength (mM)	k_1 ($M^{-1} \cdot s^{-1}$) ($\times 10^{-4}$)
Cytochrome c_1^{3+} + ascorbate^{2-}	30	4.6
	1100	16.4
Cytochrome c_1^{3+} + TMPD	107	3.8
	1100	3.8
Cytochrome c_1^{2+} + $Fe(CN)_6^{3-}$	107	7.2
	1100	37.0
Cytochrome c_1^{2+} + $Co(phen)_3^{3+}$	107	125
	1100	2.6

The second-order rate constant (k_1) for these redox reactions of cytochrome c_1 was determined from the slope of the straight lines. The various values of the second-order rate constants at the two ionic strengths are presented in Table I.

It is clear that an increase in ionic strength resulted in an increase in the value of k' for the reaction of cytochrome c_1 with ascorbate (Fig. 2A) or with $Fe(CN)_6^{3-}$ (Fig. 2B), whereas a decrease is observed for the oxidation of cytochrome c_1 by $Co(phen)_3^{3+}$ (Fig. 2C). These results indicate that at pH 7.0 cytochrome c_1 behaves as a negatively charged particle. As expected, no ionic-strength dependence is observed at neutral pH for the reaction of cytochrome c_1 with the uncharged TMPD molecule (Fig. 2D).

The ionic-strength dependence of the reactions of cytochrome c_1 with ascorbate, $Fe(CN)_6^{3-}$ and $Co(phen)_3^{3+}$ (pH 7.0) was studied in more detail and the results of these experiments are presented in Fig. 3A–C. In these figures the second-order rate constant was plotted as a function of the ionic strength. A linear relationship was observed for the three redox reactions of cytochrome c_1. The slopes of the straight lines are a measure of the effective net charges of protein (Z_A) and reagent (Z_B).

Extrapolation of these lines to zero ionic strength yields values for the second-order rate constants ($k_{I=0}$) where screening effects by ions are absent. The values for $Z_A \cdot Z_B$ and $k_{I=0}$ for the reaction of cytochrome c_1 with ascorbate, $Fe(CN)_6^{3-}$ and $Co(phen)_3^{3+}$ are summarized in Table II. From the data of the ionic-strength dependence of the

TABLE II

IONIC-STRENGTH STUDIES OF THE REACTION OF CYTOCHROME c_1 WITH NON-PHYSIOLOGICAL REDOX AGENTS

Conditions: potassium phosphate (pH 7.0), 1% Tween 20.

Reaction	$Z_A Z_B$	$k_{I=0}$ ($M^{-1} \cdot s^{-1}$)
Cytochrome c_1^{3+} + ascorbate^{2-}	+8	$1.4 \cdot 10^4$
Cytochrome c_1^{2+} + $Fe(CN)_6^{3-}$	+14	$3.2 \cdot 10^3$
Cytochrome c_1^{2+} + $Co(phen)_3^{3+}$	−16	$1.3 \cdot 10^8$

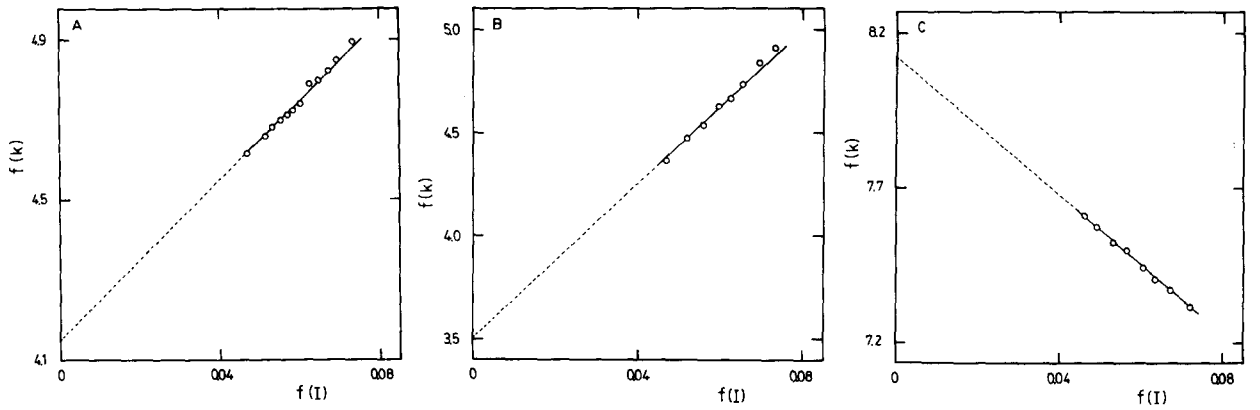

Fig. 3. Effect of ionic strength on the second-order rate constants of the reaction between cytochrome c_1 and non-physiological redox agents. Conditions: potassium phosphate (pH 7.0), 1% Tween 20. Changing of the ionic strength was accomplished by increasing the concentration of the buffer or by the addition of KCl. (A) The reaction between ferricytochrome c_1 (1.95 μM) and ascorbate (2 mM), $f(k)=\log k+2\sqrt{I}/(1+1.31\sqrt{I})$ and $f(I)=\sqrt{I}/(1+7.4\sqrt{I})$. (B) The reaction between ferrocytochrome c_1 (0.95 μM) and $K_3Fe(CN)_6$ (250 μM), $f(k)=\log k+4.5\sqrt{I}/(1+1.47\sqrt{I})$ and $f(I)=\sqrt{I}/(1+7.4\sqrt{I})$. (C) The reaction between ferrocytochrome c_1 (0.95 μM) and $Co(phen)_3^{3+}$ (10 μM), $f(k)=\log k+4.5\sqrt{I}/(1+2.29\sqrt{I})$ and $f(I)=\sqrt{I}/(1+7.4\sqrt{I})$.

reaction of ferrocytochrome c_1 with $Fe(CN)_6^{3-}$ (Fig. 3B) and with $Co(phen)_3^{3+}$ (Fig. 3C), $Z_A \cdot Z_B$ values of +14 and −16, respectively, were determined. From these values a net charge of −5 for the reduced enzyme was calculated. For the reaction of ferricytochrome c_1 with ascorbate a $Z_A \cdot Z_B$ value of +8 was found. Since the net charge of the reactive ascorbate species is −2, this gives a net charge of −4 for oxidized cytochrome c_1. The difference in net charge between ferri- and ferrocytochrome c_1 corresponds with the value based on the difference in valency state of the haem iron [6].

Discussion

The data on the pH dependence of the reduction of cytochrome c_1 by ascorbate show that at neutral and alkaline pH this protein is mainly reduced by the doubly deprotonated form of ascorbate. The same result has also been found for cytochrome *c*, cytochrome c_1, cytochrome *c*-551, azurine and cytochrome *c*-552 [14]. Additional evidence that at neutrality the doubly negatively charged ascorbate ion is the predominant reductive species for cytochrome *c* was obtained from the study of the ionic-strength dependence of the ascorbate reduction of native and CDNP-lysine cytochrome *c* derivatives [15]. The results of these studies have demonstrated a decrease in the charge of the native protein by two units — the product of the net charges of cytochrome *c* and ascorbate diminishes by 4 at pH 7.8. This implies that the doubly deprotonated species of ascorbate is the main reductant for cytochrome *c* at neutral and alkaline pH.

The enhancement in the rate of reduction of cytochrome c_1 was ascribed to the consequence of a conformational or ligand-exchange process in the protein [16]. This conclusion was based upon the bleaching of the 690 nm absorption band of cytochrome c_1 at higher pH, a phenomenon which is considered to be indicative of substitution of a haem-iron ligand (probably methionine). This replacement would result in a better accessibility of the haem iron for ascorbate. This explanation is unlikely, since similar pH profiles for the reduction by ascorbate have been found for plastocyanine [17] and azurine [14], two non-haem blue copper proteins.

The study of the kinetics of the reaction of cytochrome c_1 with ascorbate, $Fe(CN)_6^{3-}$ and $Co(phen)_3^{3+}$ showed that electrostatic interactions govern the rate of these reactions. Furthermore, it was concluded from these experiments that at neutral pH cytochrome c_1 behaves as a negatively charged particle. This conclusion was also drawn

from the data of the ionic-strength dependence of the reaction between cytochrome *c* and cytochrome c_1 [2,18] and from the value of the isoelectric point (pH 5.5) of cytochrome c_1 [19].

Application of the Debye-Hückel theory to the ionic-strength dependence of the redox reactions of cytochrome c_1 with small redox agents made it possible to determine the charge of this protein (oxidized enzyme −4, reduced enzyme −5) involved in these redox reactions. By studying the kinetics of chemically modified derivatives of cytochrome *c* with non-physiological redox agents, it was shown (Ref. 15; and unpublished results) that the overall charge of the protein and not a local charge determines the rate of the reaction. For horse cytochrome *c* [20–22], *Euglena gracilis* cytochrome *c*-552 [20], *Rhodospirullum rubrum* cytochrome c_2 [21] and *Chromatium vinosum* Hipip [22], the experimentally determined charge of the protein, using the Brønsted-Debye-Hückel equation, was found to be in close agreement with the net charge calculated from the amino acid sequence of these proteins.

This is also the case when the kinetically determined charge of cytochrome c_1 was compared with the net charge determined from the amino acid sequence of bovine cytochrome c_1 [23]. For the calculation of the charge of cytochrome c_1 from its sequence it was assumed that at neutral pH the following amino acids contribute to the net charge of the protein: 13 aspartic acid, 16 glutamic acid, 12 lysine and 15 arginine residues. Furthermore, charges of −2 for both propionic carboxyl groups of the haem, +1 for the ferrihaem, −1 for the carboxyl and +1 for the amino terminus were taken into account. If the histidine residues are uncharged, a net charge for ferricytochrome c_1 of −3 can be calculated. This value is close to the charge of −4 for the oxidized enzyme, determined from the reduction of the protein by ascorbate.

Apparently, the histidine residues do not contribute to the charge of the protein, since in the pH range 6–8 little effect of pH was found on the value of the k_1 of the reaction between ferrocytochrome c_1 and $Fe(CN)_6^{3-}$ (unpublished results). This implies that most histidine residues of cytochrome c_1 do not have a pK_a value in this pH range, which is possible but rather unusual [24,25].

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