Biochimica et Biophysica Acta, 376 (1975) 285–297

BBA 46871

THE MECHANISM OF REDUCTION OF CYTOCHROME *c* AS STUDIED BY PULSE RADIOLYSIS

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(Received July 5th, 1974)

SUMMARY

1. The reaction of hydrated electrons with ferricytochrome *c* was studied using the pulse-radiolysis technique.

2. In 3.3 mM phosphate buffer (pH 7.2), 100 mM methanol and at a concentration of cytochrome *c* of less than 20 μM the reduction kinetics of ferricytochrome *c* by hydrated electrons is a bimolecular process with a rate constant of $4.5 \cdot 10^{10}$ $M^{-1} \cdot s^{-1}$ (21 °C).

3. At a concentration of cytochrome *c* of more than 20 μM the apparent order of the reaction of hydrated electrons with ferricytochrome *c* measured at 650 nm decreases due to the occurrence of a rate-determining first-order process with an estimated rate constant of $5 \cdot 10^6$ s^{-1} (pH 7.2, 21 °C).

4. At high concentrations of cytochrome *c* the reaction-time courses measured at 580 and 695 nm appear to be biphasic. A rapid initial phase (75 % and 30 % of total absorbance change at 580 and 695 nm, respectively), corresponding to the reduction reaction, is followed by a first-order change in absorbance with a rate constant of $1.3 \cdot 10^5$ s^{-1} (pH 7.2, 21 °C).

5. The results are interpreted in a scheme in which first a transient complex between cytochrome *c* and the hydrated electron is formed, after which the heme iron is reduced and followed by relaxation of the protein from its oxidized to its reduced conformation.

6. It is calculated that one of each three encounters of the hydrated electron and ferricytochrome *c* results in a reduction of the heme iron. This high reaction probability is discussed in terms of charge and solvent interactions.

7. A reduction mechanism for cytochrome *c* is favored in which the reduction equivalent from the hydrated electron is transmitted through a specific pathway from the surface of the molecule to the heme iron.

INTRODUCTION

Recent studies [1–5] on the structure and mechanism of action of cytochrome *c* have identified several amino acid residues essential for the electron-transport function of the enzyme. The fact that the heme prosthetic group is nearly completely buried inside the protein moiety [1–3] and the observation that binding of specific antibodies [4] or chemical modification [5] of certain amino acids can block the oxidation of the enzyme without affecting the reduction and vice versa, have led to the conclusion that electrons can reach the heme iron only through a specific electron pathway. Two pathways are proposed: a reductive one from the cytochrome bc_1-binding site to the heme iron and an oxidative one from the heme to the cytochrome *c* oxidase-binding site [4, 5]. For the transmission of electrons to the heme it is suggested that the π-system of some aromatic amino acids is involved. In a previous communication [6] we presented evidence for the existence of a reductive pathway in ferricytochrome *c* in which Tyr-67 plays an essential role. The importance of this amino acid for the reduction mechanism of cytochrome *c* has recently been confirmed by Margoliash et al. [5] who studied the enzymic properties of Tyr-67 modified cytochrome *c* in the succinate-cytochrome *c* reductase system. In contrast with the two-pathway model Salemme et al. [7] have proposed that the electron-accepting and electron-donating site of cytochrome *c* are the same.

The X-ray crystallographic studies of Dickerson and coworkers on the structure of the oxidized horse [2] and reduced tuna [3] cytochromes *c* show that the change in redox state of the heme iron is accompanied by a change in conformation of the enzyme. According to Takano et al. [3] these conformational transitions play a fundamental role in the mechanism of action of cytochrome *c*. It is not known yet how fast such structural changes are, but if they are involved in the enzymic electron-transfer mechanism of cytochrome *c*, the rate of relaxation between the ferric and the ferrous conformation should be at least $10^3\ s^{-1}$, the rate at which cytochrome *c* undergoes oxidation and reduction in the mitochondrial electron-transport chain [8].

Pulse radiolysis [9, 10] has the advantage above rapid-mixing techniques that the reducing agent, hydrated electron (e_{aq}^-), can be generated in the relatively short time of 0.55 μs and that the reaction of e_{aq}^- with ferricytochrome *c* is extremely fast [6, 11–13] so that the heme iron can be reduced in a few microseconds. Spectral events following the rapid reduction of the heme may thus be studied in any time interval between 10^{-6} and 10^2 s.

In the reduction mechanism of cytochrome *c* as proposed by Takano et al. [3] and supported by Margoliash et al. [5] the reduction of the heme precedes the conformational change of the protein. During this structural change the positions of some amino acids in the near vicinity of the heme iron undergo marked changes. It is, therefore, likely that the ligand field around the heme iron will be affected by the conformational change and that, as a consequence, the relaxation of the protein part of the enzyme after the valence change of the iron will be accompanied by spectral changes.

Changes in absorbance following the rapid reduction of the heme iron have indeed been observed [11–14]: Land and Swallow [11], Pecht and Faraggi [12] and Lambeth et al. [14] report on a process with a first-order rate constant of about $10\ s^{-1}$. The first two groups ascribe the spectral change to a conformational change of the

enzyme while in the opinion of the latter group it represents a replacement of an iron ligand. The low rate of this reaction with respect to that of the electron flow in the electron-transport chain ($10^3\ s^{-1}$) makes it unlikely that the process is of biological importance. In this respect the process reported by Lichtin et al. [13] ($k \approx 10^5\ s^{-1}$) and attributed by them to a conformational change in the enzyme, is more apt to reflect a functional property of cytochrome *c*.

These differences in the value of the rate constants of the sequential processes and the fact that some authors report an increase of absorbance [12] at wavelengths where others find a decrease [11, 14] have prompted us to investigate the mechanism of reduction of cytochrome *c* by hydrated electrons.

Part of this work has been described before [15, 16].

MATERIALS AND METHODS

Enzyme

Monomeric horse-heart cytochrome *c* was isolated as described by Margoliash and Walasek [17]. The final preparation, chromatographed on Amberlite CG 50 (particle diameter 40–60 μm) was exhaustively deionized on mixed-bed columns, lyophilyzed and stored at −20 °C. Fully oxidized or reduced preparations were obtained by Sephadex-G 25 chromatography of $K_3Fe(CN)_6$ or ascorbate-treated samples, respectively. The concentration of the enzyme was determined by absorption spectroscopy using either $\Delta A_{550\ nm}$ (reduced minus oxidized) of 21.1 $mM^{-1} \cdot cm^{-1}$ [18] or $\Delta A_{416\ nm}$ (reduced minus oxidized) of 57 $mM^{-1} \cdot cm^{-1}$ [19].

Pulse radiolysis

The experimental set-up was as described before [19, 20]. By variation of the pulse length (10–550 ns) and the pulse dose (50–1500 rad) the initial concentration of e_{aq}^- could be varied between 0.1 and 3 μM. The concentration of e_{aq}^- was always less than 10 % of that of cytochrome *c*.

Methanol in a molar excess of at least 10^4 is used as ·OH scavenger since it is most effective in scavenging both ·OH and ·H radicals [15]. Absorbance readings were corrected for effects of slit width and the monochromator settings were calibrated against ferrocytochrome *c*.

Chemicals

The chemicals used, were of analar grade and purchased mainly from British Drug Houses.

RESULTS

Figs 1A, 1B and 1C show the reaction-time courses and the corresponding semi-logarithmic plots for the decay of e_{aq}^- absorbance at 700 nm and the formation of reduced cytochrome *c* at 416 nm, respectively. First-order reaction kinetics are observed which is to be expected since the concentration of cytochrome *c* is much larger than that of e_{aq}^-.

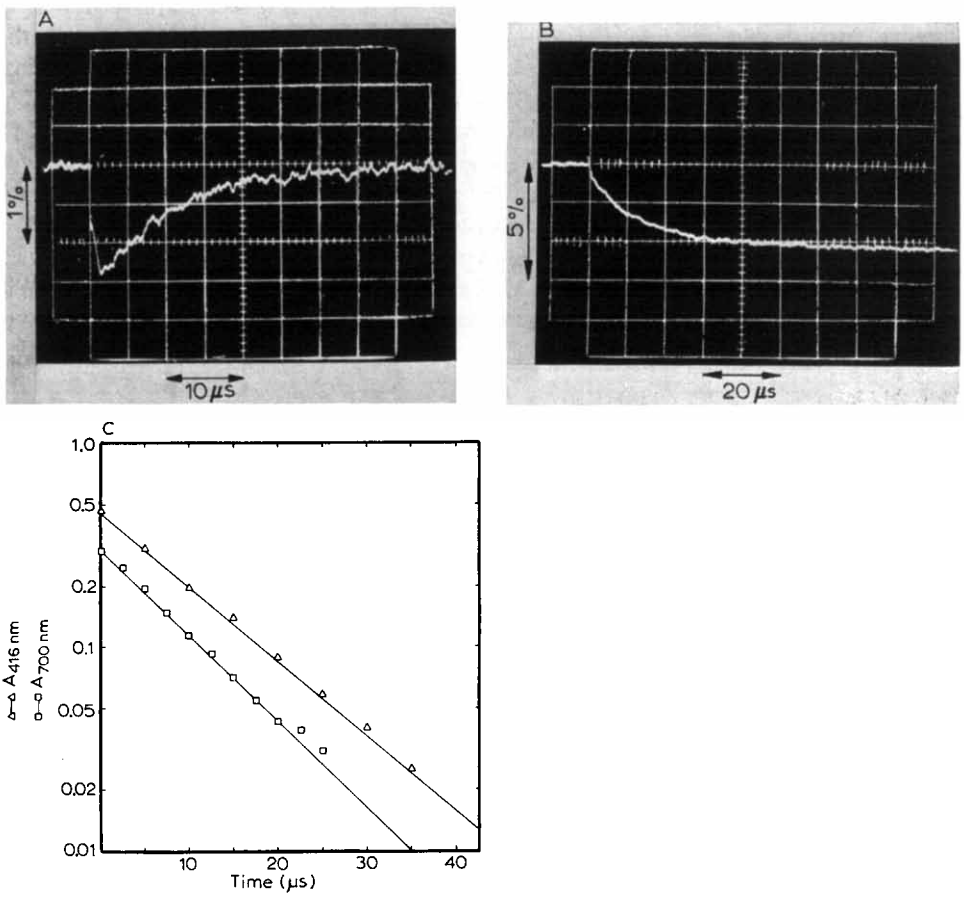

Fig. 1. Transmittance changes due to the reaction of e_{aq}^- with ferricytochrome *c*. (A) Decrease of the concentration of e_{aq}^- measured at 700 nm. (B) Reduction of cytochrome *c* measured at 416 nm. (C) First-order plots calculated from the traces shown in Figs A and B. The initial concentrations of e_{aq}^- and of ferricytochrome *c* are 0.2 and 2.4 μM, respectively; matrix solution, 3.3 mM phosphate buffer (pH 7.2) and 100 mM methanol in triple-distilled water; temperature, 21 °C.

The disappearance of e_{aq}^- is due to its reaction with the matrix solution and to its reaction with the protein. From the half-life (τ) of e_{aq}^- in the absence and presence of the protein (τ_m and τ_{m+c}, respectively) the half-life for the reaction of e_{aq}^- with the protein τ_c can be calculated [21] from Eqn 1.

$$\tau_c = \tau_{m+c}\left\{1 - \frac{\tau_{m+c}}{\tau_m}\right\}^{-1} \quad (1)$$

τ_c can be used for the calculation of the second-order rate constant of the reaction of e_{aq}^- with the enzyme molecules as well as for the determination of the amount of hydrated electrons that have reacted with the protein:

$$[e_{aq}^-]_c = \left\{1 - \frac{\tau_{m+c}}{\tau_m}\right\}[e_{aq}^-]_t \quad (2)$$

Here, $[e_{aq}^-]_c$ represents the concentration of hydrated electrons reacting with cytochrome *c* and $[e_{aq}^-]_t$ the total concentration of hydrated electrons generated in the solution.

The difference between the observed half-life (τ_{m+c}) and the real half-life (τ_c) depends on the concentration of cytochrome *c* and approaches zero at high concentrations of the proteins. Under our conditions (1–60 μM cytochrome *c*) 3.3 mM phosphate buffer (pH 7.2), 0.1–0.5 M methanol, the correction is 20.1 %.

The final product of the reaction of e_{aq}^- with ferricytochrome *c* is spectroscopically indistinguishable [11–13] from ferrocytochrome *c* and therefore the change in absorbance induced by a certain radiation dose absorbed can be used for the calculation of the concentration of cytochrome *c* reduced per unit dose absorbed. Since the concentration of e_{aq}^- produced per unit dose absorbed is known [17], the amount of e_{aq}^- reacting with cytochrome *c* can be calculated from Eqn 2. From these data the yield of the heme reduction (σ), which is defined as the ratio (given in percent) of the amount of reduced cytochrome *c* to the amount of e_{aq}^- reacting with cytochrome *c*, can be obtained. Some values for σ are summarized in Table I. It is clear from the table that σ is independent of both the concentration of e_{aq}^- and the concentration of cytochrome *c*, but that it is markedly dependent on the pH of the medium.

TABLE I

Some values of the yield of reduction (σ) for the reaction of e_{aq}^- with ferricytochrome *c*, at pH 7.2 and pH 11.0. Matrix solution: 3.3 mM phosphate (pH 7.2) or 1 mM NaOH (pH 11.0) and 100 mM methanol in triple-distilled water; buffer temperature 21–22 °C. The final absorbance was read 50–100 μs after the pulse.

$[c^{3+}]$ (μM)	$[e_{aq}^-]$ (μM)	σ (%)*	
		pH 7.2	pH 11.0
5.5	0.3	85±5	12±3
5.5	3.2	75±5	15±3
10.2	0.8	80±5	20±5
10.2	3.3	75±5	15±4

* These values include the contribution to the reduction (approx. 2 %) due to the reaction of cytochrome *c* with unscavenged ·H radicals [15].

The effect of the proton concentration on σ is shown in Fig. 2. At about neutral pH, σ has a maximal value of 85 %, while upon decreasing or increasing the pH of the matrix solution the yield of the heme *c* reduction diminishes. At pH 5.8, the yield is about 65 % while at pH 10.5 less than 15 % of the hydrated electrons that have reacted with cytochrome *c* is capable in reducing the heme iron. Nitration of Tyr-67 considerably decreases the σ of the reaction. At neutral pH σ is about 30 %. In contrast to what is observed for native cytochrome *c* no optimum is observed in σ for nitrocytochrome *c*. The increase of the value of σ at lowering the pH may be due to restoration of the "native" conformation induced by protonation of the nitrotyrosil ion (see ref. 6).

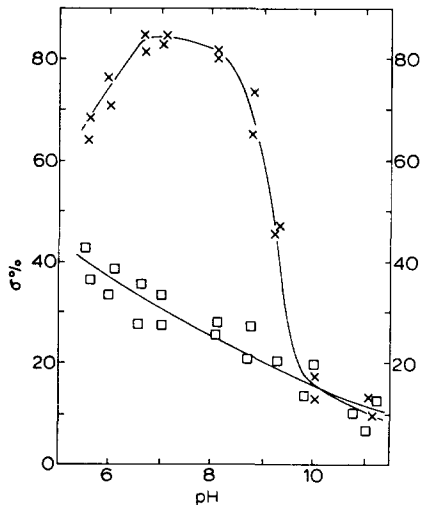

Fig. 2. The reduction yield σ as a function of the pH for the reaction of e_{aq}^- with ferricytochrome *c* (× — ×) and ferrinitrocytochrome *c* (□—□). Cytochrome *c*, 5–6 μM; e_{aq}^-, 0.2–0.4 μM; at pH 6–8, phosphate; at pH above 8, borate plus carbonate; buffer, 3.3 mM and methanol, 0.1 M, in triple-distilled water; temperature, 21–22 °C. Nitrocytochrome *c* was prepared as mentioned before [6]; $\Delta A_{416\ nm}$ was measured 50–100 μs after the pulse.

A value of σ less than 100 % indicates that not all of the hydrated electrons reacting with ferricytochrome *c* have reduced heme irons. Other reactive sites must therefore be present. At low pH, reaction of e_{aq}^- with the very reactive histidyl H^+ residue [22] dominates, whereas at high pH, where cytochrome *c* itself is much less reactive [6], the majority of the hydrated electrons are likely to react with the aromatic side-chains of amino acids. In order to investigate whether these side reactions affect the enzymic properties of cytochrome *c* we have measured the activity of irradiated cytochrome *c* in the ascorbate-TMPD-cytochrome *c*-cytochrome *c* oxidase-O_2 system [23, 24]. After radiation doses, yielding amounts of e_{aq}^- equal to the amounts of cytochrome *c* present (resulting in about 80 % reduced cytochrome *c*), no significant decrease in enzymic activity was found (not shown). Hence, the reaction of e_{aq}^- with oxidised cytochrome *c* in our experiments does not affect the biological properties of cytochrome *c*.

Since the iron in cytochrome *c* is buried in the protein moiety [2, 3], a reductive pathway along which electron transmission occurs, should exist in cytochrome *c*. In an

TABLE II

The second-order rate constants $k_{650\ nm}$ and $k_{416\ nm}$ for the reaction of e_{aq}^- with ferricytochrome c, at pH 7.2 and pH 11.0. For experimental conditions: see Table I.

$[c^{3+}]$ (μM)	$[e_{aq}^-]$ (μM)	pH	$k_{650\ nm} \times 10^{-10}$ ($M^{-1} \cdot s^{-1}$)	$k_{416\ nm} \times 10^{-10}$ ($M^{-1} \cdot s^{-1}$)
5.5	0.3	7.2	4.1±0.2	4.2±0.3
10.2	0.5	7.2	3.9±0.3	4.0±0.3
5.5	0.3	11.0	0.9±0.1	1.0±0.1
10.2	0.6	11.0	0.7±0.1	0.6±0.1

attempt to estimate the rate of such a process we have compared the rate of reduction of the heme iron with the rate of reaction of e_{aq}^- with cytochrome *c*. The apparent first-order rate constants for both reactions determined under various conditions are shown in Table II.

It is evident that the rate ($k = 4.1 \cdot 10^{10}\ M^{-1} \cdot s^{-1}$ at pH 7.2) at which e_{aq}^- disappears (650 nm) equals the rate of heme reduction (416 nm) (cf. ref. 11). Consequently, if electron transmission exists in cytochrome *c*, under the conditions used in Table II, the rate of this process is much greater than that in the binding of e_{aq}^- to cytochrome *c*. In order to enhance the rate of the binding reaction, we have increased

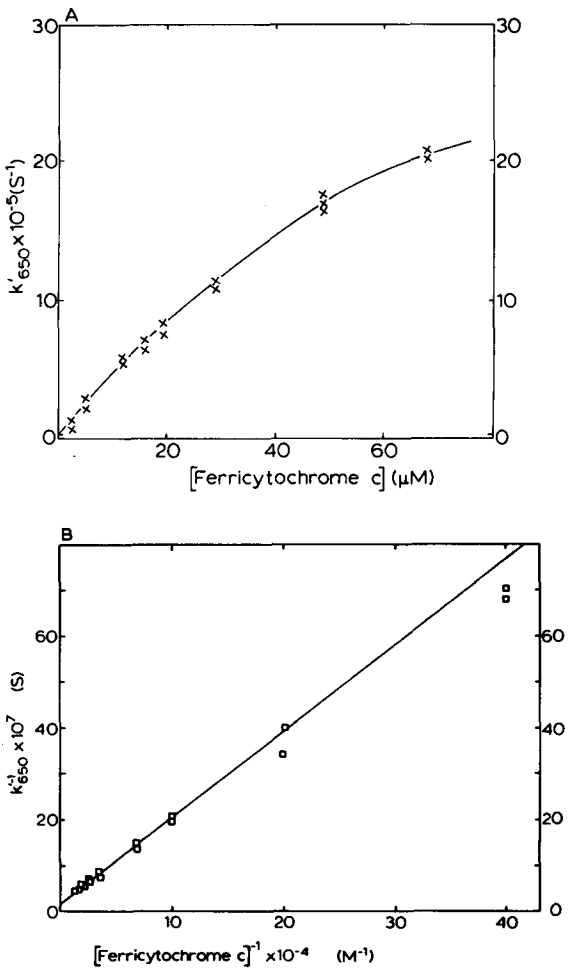

Fig. 3. The pseudo-first-order rate constant observed at 650 nm ($k'_{650\ nm}$) for the reaction of e_{aq}^- with ferricytochrome *c* at different cytochrome *c* concentrations. (A) Dependence of $k'_{650\ nm}$ of the concentration of ferricytochrome *c*. (B) Double reciprocal plot of $k'_{650\ nm}$ and the ferricytochrome *c* concentration. Cytochrome *c*, 2–70 μM; e_{aq}^-, 0.2–3.3 μM; methanol, 0.1 M for cytochrome *c* concentrations less than 10 μM; at higher concentrations of cytochrome *c* the methanol concentration is increased proportionally; temperature, 21–22 °C.

the concentration of cytochrome *c*. Since at high cytochrome *c* concentrations (>10 μM) it is hard to follow the rate of reduction of the heme iron, due to its high absorbance in the γ-band region, the disappearance of the hydrated electron has been studied.

In Fig. 3A the observed pseudo-first-order rate constant for the reaction of e_{aq}^- with ferricytochrome *c* ($k' = k_1 \cdot$ [cytochrome *c*]$+k_{-1}$) is plotted as a function of the protein concentration. At low concentrations of cytochrome *c*, k' appears to be linear to the enzyme concentration, indicating that under these conditions the reaction of e_{aq}^-, with ferricytochrome *c* can be described as a simple bimolecular process, with a rate constant (k_1) of $4.5 \cdot 10^{10}$ $M^{-1} \cdot s^{-1}$ (pH 7.2) consistent with the results of Table II. The apparent dissociation rate constant (k_{-1}) can be estimated from the intercept with the ordinate and has a value with an upper limit of 10^4 s^{-1}. At a protein concentration higher than 20 μM, however, the line becomes curved, indicating the presence of an intermediate in the reaction. Hence, the results can be explained by the following sequence of events:

$$e_{aq}^- + c^{3+} \xrightarrow{k_1} [e_{aq}^- \ldots c^{3+}]^\star \xrightarrow{k_2} c^{2+} \qquad (3)$$

At low concentration of cytochrome *c*, k_1 is rate-determining, while at higher concentrations k_2 becomes rate-determining and the value of k' will approach the value of k_2. Our present experimental set-up does not allow us to use protein concentrations higher than 70 μM, since then the half-lives of the reaction become shorter than the pulse time.

An estimate of k_2 can be obtained from Fig. 3B where the results of Fig. 3A are plotted double reciprocally. From the intercept of the line with the ordinate, which represents the inversed value of k' at infinite cytochrome *c* concentration, k_2 is calculated to be $5 \cdot 10^6$ s^{-1} (21 °C, pH 7.2).

At high concentrations of ferricytochrome *c* (>30 μM) the reaction-time course for the reduction of cytochrome *c* measured at 580 nm is biphasic (see Fig. 4A). The initial phase, which is complete within the time of the disappearance of the hydrated electrons, is rather complex since both e_{aq}^- and cytochrome *c* contribute markedly to the absorbance change. The second phase, in which the absorbance further decreases, is due to a strictly first-order process. The rate constant for this reaction which is independent of cytochrome *c* concentration (see Fig. 4C) has a value of $1.3 \cdot 10^5$ s^{-1} (pH 7.2, 21 °C).

It is generally accepted, that the 695-nm absorbance of cytochrome *c* is characteristic for the native conformation of the oxidized molecule and therefore the reaction of e_{aq}^- with ferricytochrome *c* has also been studied at 695 nm. Similarly as at 580 nm the reaction-time course at 695 nm is biphasic (Fig. 4B). The rate constant for the second phase, which is independent of the cytochrome *c* concentration (Fig. 4C), has a value of $1.2 \cdot 10^5$ s^{-1} (pH 7.2, 21 °C), which is similar to that observed for the slow phase at 580 nm (Fig. 4A). The relative contributions of the rapid and the slow phase determined at 580 and 695 nm, however, differ considerably. At the end of the initial phase the absorbance of the solution at 580 nm has decreased to about 30 % of the total absorbance change, while at 695 nm 75 % of the total absorbance change remains. With a σ of 80 % and an optical path of 2 cm, a $\Delta A_{695\,nm}$ (reduced minus oxidized) of 0.8 ± 0.1 $mM^{-1} \cdot cm^{-1}$ is calculated and it is concluded that about 75 % of the $\Delta A_{695\,nm}$ is brought about by change in conformation of the protein while the

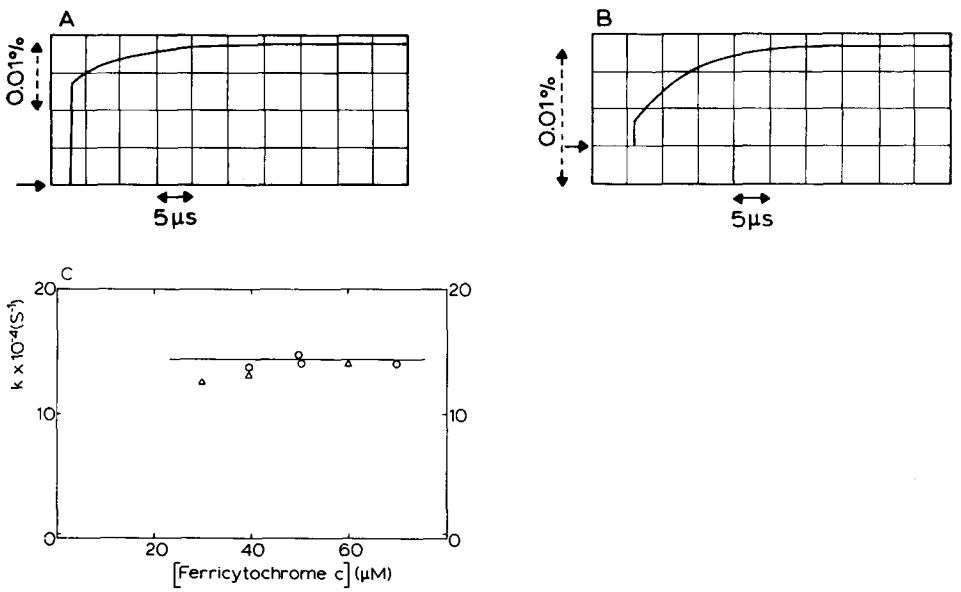

Fig. 4. Kinetics of the reaction following the reduction of the heme iron. (A) Transmittance change at 580 nm. Cytochrome *c* and e_{aq}^-, 50 and 2.2 μM, respectively. (B) Transmittance changes at 695 nm. Cytochrome *c* and e_{aq}^-, 60 and 2.3 μM, respectively. (C) Dependence of the first-order rate constant on the concentration of ferricytochrome *c*; ○—○, 580 nm; and △—△, 695 nm. Matrix solution: 3.3 mM phosphate buffer (pH 7.2) and 500 mM methanol in triple-distilled water; temperature, 21 °C. Note: arrows indicate levels of transmittance before the electron pulse.

remaining 25 % is due to the primary effect of the ferric-ferrous transition on the ligand field of the heme iron.

The possibility that the $\cdot CH_2OH$ radical, produced in the reaction of methanol with ·OH, causes an additional reduction of the enzyme should be considered. Therefore, the scavenger methanol was replaced by formate. With 0.1 M formate and 40 μM ferricytochrome *c* also a biphasic change in absorbance was observed at 580 nm (not shown). Although the reduction reaction is somewhat slower ($k = 3.3 \cdot 10^{10}\ M^{-1} \cdot s^{-1}$) than with methanol, the rate constants for the second process ($k = 1.3 \cdot 10^5\ s^{-1}$) are identical. This finding is in agreement with the results of Lichtin et al. [13] who found for *tert*-butanol as scavenger, the same rate ($1.2 \cdot 10^5\ s^{-1}$) for this process. The independence of this rate constant on the nature of the ·OH scavenger and the fact that the relative contribution to the absorbance by the second process is wavelength-dependent, seem to rule out the possibility that the second process represents a further reduction of cytochrome *c* by $\cdot CH_2OH$ radicals.

Apart from the relatively rapid reactions described above, at neutral pH and cytochrome *c* concentrations between 4 and 10 μM, small changes (2–4 % of the total absorbance change) are observed at 416 nm (not shown) with a rate constant of $2 \cdot 10^3$–$4 \cdot 10^3\ s^{-1}$. So far we have not attempted to elucidate further the nature of this process.

Under the conditions used in our pulse-radiolysis studies (3.3 mM phosphate buffer (pH 7.2), 100 mM methanol, freshly isolated monomeric cytochrome *c*, 22 °C)

other changes with rate constants of about $10\ s^{-1}$ (cf. refs 11, 12, 14) have not been observed by us.

DISCUSSION

Our results can be summarized in the following scheme:

$$e_{aq}^- + c^{3+} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} [e_{aq}^- \cdots c^{3+}]^* \xrightarrow{k_2} \boxed{c^{2+}} \xrightarrow{k_3} (c^{2+}) \qquad (4)$$

with $k_1 = 4.5 \cdot 10^{10}\ M^{-1} \cdot s^{-1}$; $k_{-1} < 10^4\ s^{-1}$; $k_2 = 5 \cdot 10^6\ s^{-1}$; $k_3 = 1.3 \cdot 10^5\ s^{-1}$.

First a transient complex is formed between cytochrome *c* and the hydrated electron. In the second step the electron of e_{aq}^- reduces the heme iron and finally the protein conformation changes from the oxidized to the reduced form.

Step I

At concentrations of ferricytochrome $c < 20\ \mu M$ the formation of the complex is rate-determining and the reaction between e_{aq}^- and cytochrome *c* is a second-order process. The second-order rate constant for the reaction of e_{aq}^- with cytochrome *c* determined from the slope of the curve in Fig. 3A at zero concentration is $4.5 \cdot 10^{10}\ M^{-1} \cdot s^{-1}$. This value is of the same order of magnitude as those reported in the literature ($2 \cdot 10^{10}$–$10 \cdot 10^{10}\ M^{-1} \cdot s^{-1}$, refs 11–13). The differences in the reported values can be partly ascribed to differences in the ionic strength of the matrix solutions employed. Due to the high positive charge on the cytochrome *c* molecule at pH 7, the effect of ionic strength on the rate constant is considerable even for mmolar buffer concentrations. It can be calculated (cf. ref. 20) that at neutral pH and in the absence of buffer the rate constant is 2.3 times that in 3.3 mM phosphate buffer (same pH). This is illustrated in Table III where measured rate constants are presented for cytochrome *c* in the presence and absence of added salt. Since the second-order rate constant is hardly dependent on temperature (unpublished), differences remaining after corrections for ionic strength are probably due to differences in enzyme preparation.

TABLE III

Effect of matrix composition on the second-order rate constant of the reaction of e_{aq}^- with ferricytochrome *c*. The rate constants were measured at neutral pH and ambient temperatures (20±2 °C).

Ref.	$k \times 10^{-10}\ (M^{-1} \cdot s^{-1})$		
	0.1 M salt	3.3 mM phosphate	triple-distilled water
Land and Swallow [11]	2[a]	—	—
Pecht and Faraggi [12]	—	—	10
Lichtin et al. [13]	2.4[b]	—	6
Wilting (unpublished)	—	—	10.5
This paper	3.3[a]	4.1–4.5	(9.5–10.5)[c]

[a] Sodium formate.
[b] $NaClO_4$.
[c] Calculated from the *k* values in 3.3 mM phosphate with the Debye-Brønsted equation [20].

In our hands commercially available cytochrome *c* preparations, used without further purification, were less active towards e_{aq}^- (25–30 %) than freshly prepared monomeric samples of cytochrome *c*. Thus, at neutral pH and at very low ionic strength, the rate constant for the reaction of e_{aq}^- with ferricytochrome *c* is approximately 10^{11} $M^{-1} \cdot s^{-1}$. This very high value suggests that the reaction rate approaches that of a diffusion-controlled process.

In general the rate constant of a bimolecular reaction depends on the number of encounters and a probability factor:

$$k = N \cdot \Sigma \cdot \Phi \qquad (M^{-1} \cdot s^{-1}) \tag{5a}$$

where N is the Avogadro number, Σ the encounter rate and Φ a probability factor representing the fraction of encounters giving products. According to Smoluchowski [26] and Weston and Schwarz [27], the encounter rate for uncharged molecules can be calculated from the diffusion constants and the respective radii of the reacting species. Since both cytochrome *c* and the hydrated electron are charged the encounter frequency is affected by the electrostatic interaction between the species and by the ionic strength of the medium. Factors accounting for the contribution of the charge (f_c) and the ionic strength (f_i) have been derived by Debye [25, 27]:

$$k = N \cdot \Sigma \cdot \Phi \cdot f_c \cdot f_i \tag{5b}$$

Under the conditions of the pulse-radiolysis experiments, $N \cdot \Sigma \cdot f_c \cdot f_i = 10.2 \cdot 10^{10}$ $M^{-1} \cdot s^{-1}$ (see also ref. 20) and thus Φ is about 0.4. At neutral pH the reduction yield for the reaction of e_{aq}^- with cytochrome *c* is about 80 %. With $\Phi = 0.4$ this means that 30 % of all encounters of e_{aq}^- with the surface of the molecule lead to the formation of ferrocytochrome *c*. Since only 3 % of the surface of cytochrome *c* allows direct contact of the heme with the solvent [2] the high value of the probability factor (30 %) may be taken as an indication that e_{aq}^- reacts with an active site on the surface of cytochrome *c* and not directly with the heme. The electron is then transmitted through the protein to the heme iron. In line with the suggestion of a specific electron pathway in cytochrome *c* are our earlier findings that either nitration of Tyr-67 [6] or increase of the pH to 11 [6, 20] decrease both the rate and yield of reduction of the heme iron. The above suggestion also follows the hypothesis proposed by Takano et al. [3] that electrons enter and leave cytochrome *c* along specific pathways through the protein. Since modification of Tyr-67 inhibits the enzymic [5] as well as radiolytic [6] reduction of cytochrome *c*, it is feasible that electrons from both cytochrome bc_1 and e_{aq}^- are transmitted by the same pathway through cytochrome *c*.

The question then remains how the electron finds, with such a high probability, the entrance to this electron path. An explanation may be based on the presence of highly positively charged areas [2, 3] on the surface of the cytochrome *c* molecule which could cause the electron to interact preferentially with these sites.

In this context it is interesting to note that Takano et al. [3] and Margoliash et al. [5] propose that the entrance of the reductive pathway in cytochrome *c* is surrounded by positive charges. That such effects of charge interaction can be of importance is suggested by the very low reduction yield for the reaction of e_{aq}^- with the highly negatively charged cytochrome *c* oxidase molecule [19].

An additional reason for the high probability of reaction may be found in the

so-called "cage" theory of Frank and Rabinowitch [28] and Noyes [29, 30]. The principle of the theory, is that in contrast to what happens in the gas phase, molecules colliding in a liquid medium do not separate immediately from each other. The cage or shell of solvent molecules surrounding the reacting molecules will prevent direct escape from their inertial and/or electric attraction and thus in the liquid phase molecules encounter. During such an encounter the molecules may undergo several collisions (10–100) before they leave their interaction sphere. This effect in combination with the electric attraction by local positive charges are most likely the reason for the high probability factor (Φ) of the reaction.

Step II

Since the heme iron of cytochrome *c* is wrapped up in the protein the hydrated electron can only indirectly reach the heme iron. The rate of the iron reduction must, therefore, be limited by either the time needed for the dehydratation of e_{aq}^- or the transmission of the electron to the heme iron. Unfortunately our experimental data do not allow a choice between these two possibilities but one may speculate that transmittance of electrons through a reductive pathway is faster than the observed $5 \cdot 10^6\ s^{-1}$.

Step III

The rate of the spectral changes observed at high cytochrome *c* concentrations, following the rapid reduction of the heme of cytochrome *c* is affected neither by a 3-fold increase in the cytochrome *c* concentration (Fig. 4C) nor by the use of other ·OH scavengers (cf. Results and ref. 13). It is likely, therefore, that the distortion of the ligand field of the heme iron, induced by the change from the ferric to the ferrous conformation of the protein, causes the secondary changes in absorbance. This conclusion is supported by two observations: (1) most of the conformation-sensitive 695-nm absorbance decays in the second phase (i.e. after the iron is reduced); and (2) the rate constant for the change in conformation of hemoglobin from the R- to the T-state occurs at a similar rate ($k = 4.6 \cdot 10^4\ s^{-1}$) [31].

Our observation that reduction of the heme iron precedes the conformational change is in accordance with the mechanism for the reduction of cytochrome *c*, proposed by Takano et al. [3]. In their opinion the electron of cytochrome bc_1 that enters cytochrome *c* at Tyr-74 is transmitted directly to the aromatic ring of Trp-59. The surplus of negative charge on this amino acid residue induces by polarization a shift of electron density from Tyr-67 via the porphyrin ring to the positively charged heme iron. A conformational change following the ferric-ferrous transition brings the aromatic rings of the negative Trp-59 and the positive Tyr-67 close enough together to restore their electron neutrality. Our results indicate that this conformational change occurs with a rate of about $10^5\ s^{-1}$.

ACKNOWLEDGEMENTS

We wish to thank Dr H. Nauta for his interest and valuable discussion. Our thanks also go to Dr G. Casteleijn and Mr I. A. Raap for their help with the accelerator and to Mrs P. M. C. Harthoorn, Mr J. A. H. Hüttmann, Mr G. Wubs and Mr A. H. Dal for their assistance with the enzyme isolations and the enzymic assays. One

of us (J.W.) would like to express his thanks to the director of the Paterson Laboratories in Manchester where some of the measurements were performed and to Dr M. Ebert for his advice and valuable discussions. This work was supported, in part, by grants from the Netherlands Organization for the Advancement of Pure Research (Z.W.O.) under auspices of the Foundation for Chemical Research (S.O.N.) and for Biophysics.

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