Biochimica et Biophysica Acta, 459 (1977) 207–215

BBA 47229

MECHANISM OF THE REACTION OF HYDRATED ELECTRONS WITH FERROCYTOCHROME *c*

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(Received June 10th, 1976)

SUMMARY

1. The hydrated electron reacts with ferrocytochrome *c* to form an unstable intermediate. This intermediate decays in a first-order manner to give, in the first instance, a product which has a similar absorption spectrum in the range 400–610 nm as normal ferricytochrome *c*.

2. At 21 °C the rate constant for the reaction of hydrated electrons with ferrocytochrome *c* at pH 7.4 (2 mM phosphate buffer) is $(3.0 \pm 0.3) \cdot 10^{10}\ M^{-1} \cdot s^{-1}$. As the pH is increased above pH 8.0 the rate constant steadily decreases. The dependence of the rate constant on pH can be explained if ferrocytochrome *c* has a pK of around 9.2.

3. At 21 °C and pH 7.4, the rate constant for the decay of the intermediate is $(1.40 \pm 0.15) \cdot 10^{5}\ s^{-1}$. This reaction shows no pH dependence in the range 6.2–11.0.

4. A mechanism is proposed whereby the central metal atom of the ferrocytochrome *c* is oxidised and a thioether bond is reduced. The resulting ferricytochrome *c* species then slowly develops an absorbance at 606 nm due to the attack of the sulfhydryl group on the haem.

INTRODUCTION

The reaction of hydrated electrons with ferrocytochrome *c* has been indirectly studied by allowing ferricytochrome *c* to react with an excess of hydrated electrons produced from ^{60}Co or X-ray irradiations [1–3].

In each of these investigations it was found that on increasing the concentrations of excess hydrated electrons the absorbance at 550 nm was decreased. This decrease of absorption is due to a conversion of ferrocytochrome *c* to a green-coloured product with an absorption band at 606 nm. Quantitative data on the kinetics and spectral properties of the intermediates in this reaction are, however, lacking. We therefore started an investigation of the effects of the hydrated electrons on ferrocytochrome *c* using the pulse-radiolysis technique.

Abbreviations: e_{aq}^-, hydrated electrons; PCMB, *p*-chloromercuribenzoate.

Hydrated electrons were produced by the pulse-radiolysis of argon-saturated water; the yield (G) being taken to be 2.7 molecules per 100 eV absorbed, *i.e.* $G(e_{aq}^-) = 2.7$ [4]. Hydroxyl radicals [5] and most of the hydrogen atoms [6] were scavenged by 1.0 M tertiary butanol. When the solutions were saturated with nitrous oxide so as to replace hydrated electrons by hydroxyl radicals which then gave *tert*-butanol radicals, it was found that the spectroscopic changes were less than 3 % of those produced in the absence of nitrous oxide. This shows that *tert*-butanol radicals play a negligible part in the reactions.

METHODS AND MATERIALS

The horse heart cytochrome *c* was isolated as described previously [7]. The ferro-form was prepared by reducing the cytochrome with sodium ascorbate and then passing the solution through a Sephadex G-25 column. The concentration was determined spectrophotometrically using a difference absorption coefficient at 550 nm (red-ox) of 21.1 $mM^{-1} \cdot cm^{-1}$ [8].

All other reagents were of Analar grade except tertiary butanol which was of purissima grade from Fluka. The tertiary butanol was recrystallised four times to remove possible impurities which may shorten the life-time of the hydrated electron in the matrix solutions [9].

Two buffers were used in the investigations: Na_2HPO_4/KH_2PO_4 (2 mM) for pH values of between 6.2 and 8.4 and $H_3BO_3/NaOH$ (3 mM) for pH values between 8.6 and 10.3. The extreme alkalinity values were obtained using NaOH.

Doses (200–1200 rads, producing 0.58–3.5 μM e_{aq}^-) and concentrations of ferrocytochrome *c* (6–57 μM) were such that the reactions between e_{aq}^- and cytochrome *c* always followed simple first-order kinetics (pseudo-unimolecular).

The pulse-radiolysis apparatus was as described previously [11]. Single or double Bausch and Lomb monochromators were used with slit widths of 2.5 nm. The monochromators were calibrated using ferri- and ferrocytochrome *c*. The cells had optical path lengths of 0.9 and 2.0 cm.

RESULTS

The kinetics of the reactions

At pH 7.4 the production of hydrated electrons in the presence of excess ferrocytochrome *c* led to a first-order decrease in the absorbance of the hydrated electron at 650 nm, the rate of which was directly proportional to the cytochrome *c* concentration. The rate constant for the reaction of hydrated electrons with ferrocytochrome *c* after correcting for the loss of a small amount of hydrated electrons with the buffer solution and with the ferricytochrome *c* impurity [10] was estimated to be $(3.0 \pm 0.3) \cdot 10^{10}\ M^{-1} \cdot s^{-1}$.

The effect of pH at the range 6.2–11.0 on the reaction rate was examined using cytochrome *c* concentrations of 6–57 μM of which more than 92 % was in the reduced form. Doses were in the range of 200–1200 rads. The variation of the corrected rate constants with pH is shown in Fig. 1.

The experiments at pH 7.4 showed that there were no significant absorbance changes in the spectral range of 520–610 nm after the essential completion of the

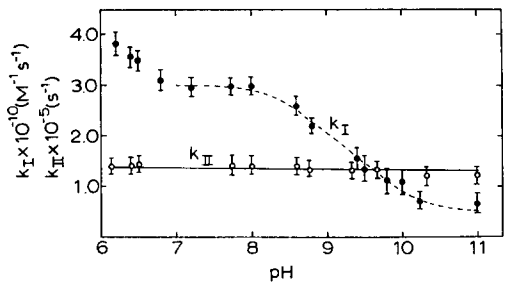

Fig. 1. Effect of pH on the rate constant of the reaction of e_{aq}^- with ferrocytochrome *c* (k_I), and of the subsequent first-order reaction (k_{II}) at 21 ±2 °C. k_I and k_{II} were calculated from the absorbance changes at 650 and 470 nm, respectively. The solutions contained $6 \cdot 10^{-6}$–$57 \cdot 10^{-6}$ M cytochrome *c* of which more than 92 % was in the reduced form and 1.0 M *tert*-butanol. The values of k_I were corrected for the reaction with the ferricytochrome *c* impurity. The dotted line is theoretical, based on a p*K* of 9.25.

reaction of the hydrated electrons with ferrocytochrome *c*. However, in the spectral range of 400–515 nm the absorbance changed considerably after completion of the hydrated electron reaction. A constant value was reached at 50 μs after the pulse. Apparently, the hydrated electron reaction (step I) is followed by another fast reaction (step II) and the spectra of the products of step I and step II are similar in the 520–610 nm range, but differ in the 400–515 nm range.

Fig. 2 shows the increase or decrease in absorbance after 50 μs, *i.e.* after completion of step I and step II. The values are corrected for the loss of electrons to the matrix solution. The figure also shows the change in absorbance which would be expected for conversion of ferrocytochrome *c* to ferricytochrome *c* with 100 % efficiency, assuming $G(e_{aq}^-) = 2.7$. The similarity in the spectral characteristics can

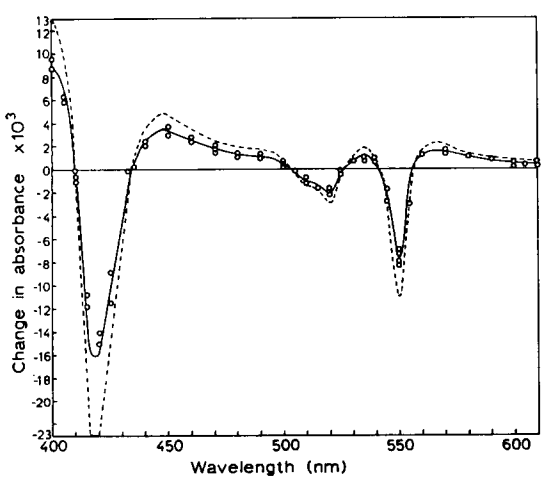

Fig. 2. Change in absorbance at approx. 50 μs after the reaction of e_{aq}^- with ferrocytochrome *c*. The points are the changes that are produced by the action of $5.77 \cdot 10^{-7}$ M e_{aq}^- in a cell of optical path length 0.9 cm. The line is calculated for 70 % oxidation; the dotted line 100 %, assuming $G = 2.7$.

be explained if it is assumed that of the aqueous electrons that react with ferrocytochrome *c*, 70 % of them lead to an oxidation of the cytochrome and the remaining 30 % give no change in the ferrocytochrome *c* spectrum in the measured spectral region.

Measurements at 470 nm showed that at pH 7.4 the change in absorbance due to step II was independent of cytochrome *c* concentration in the range: Fe^{2+} cytochrome *c* = 6.0–57 μM, Fe^{3+} cytochrome *c* = 0.24–3.0 μM, doses of 200–1200 rads and ionic strengths of 4 mM to 1.0 M (using $NaClO_4$). The effect of pH on this second reaction was examined at 470 nm using the same range of cytochrome *c* concentrations. For each pH the concentrations of cytochrome *c* were chosen so as to allow this second reaction to be reasonably separated from the hydrated electron reaction. Results at 21±2 °C are included in Fig. 1. The activation energy of this reaction was investigated in the temperature range 10.2—44 °C and was found to be 28±4 kJ/mol.

The spectrum of the intermediate, i.e. the product of step I

The overall reaction can be simply represented as:

$$e^-_{aq})_a + Fe^{2+} \text{ cyt. } c \xrightarrow{k_I} \text{Fe cyt. } c^{\neq} \xrightarrow{k_{II}} Fe^{3+} \text{ cyt. } c^* \qquad (1)$$

where $(e_{aq}^-)_a$ is that fraction of the hydrated electrons which gives a change in the absorbance of the ferrocytochrome *c* and produce the ferricytochrome *c* species. It follows from Fig. 2, $(e_{aq}^-)_a = 0.7(e_{aq}^-)_o$. The concentration of the intermediate at any time, *t*, is given by the expression:

$$[\text{Fe cyt. } c]^{\neq} = \frac{k_I^*[e^-_{aq}]_a}{k_{II} - k_I^*} (e^{-k_I^* t} - e^{-k_{II} t}) \qquad (2)$$

where $k_I^* = k_I[Fe^{2+} \text{ cyt. } c]$. If a time is chosen such that the hydrated electron reaction can be considered virtually complete the concentration of ferrocytochrome *c* removed from the solution is equal to the concentration of the reacted aqueous electrons, $[e_{aq}^-]_a$. The concentration of the ferric species produced at time *t*, is given by:

$$[Fe^{3+} \text{ cyt. } c^*]_t = [e^-_{aq}]_a - [\text{Fe cyt. } c]^{\neq}_t \qquad (3)$$

The changes in absorbance ΔA at this time are related to the optical path length 1, and the concentrations and absorbance coefficients in the solution by the expression:

$$\Delta A/1 = [\text{Fe cyt. } c]^{\neq}_t \cdot \varepsilon_{\text{Fe cyt. } c^{\neq}} + [Fe^{3+} \text{ cyt. } c^*]_t \cdot \varepsilon_{Fe^{3+} \text{ cyt. } c} - [Fe^{2+} \text{ cyt. } c] \cdot \varepsilon_{Fe^{2+} \text{ cyt. } c} \qquad (4)$$

The spectrum of the intermediate species in the range 400–520 nm was determined by delivering single pulses (350–550 rads) to ice-cooled argon-saturated solutions of ferrocytochrome *c* (10 and 16 μM, ferricytochrome *c* concentration less than 3 %), and measuring the changes in absorption at 10 and 15 μs after the pulse. After correcting for the small change in absorbance produced by the aqueous electrons reacting with the ferricytochrome *c* and the loss of some of the electrons with the matrix solutions, the concentrations of the species present at these times and hence the extinction coefficient of the intermediate were found using the above expressions. The

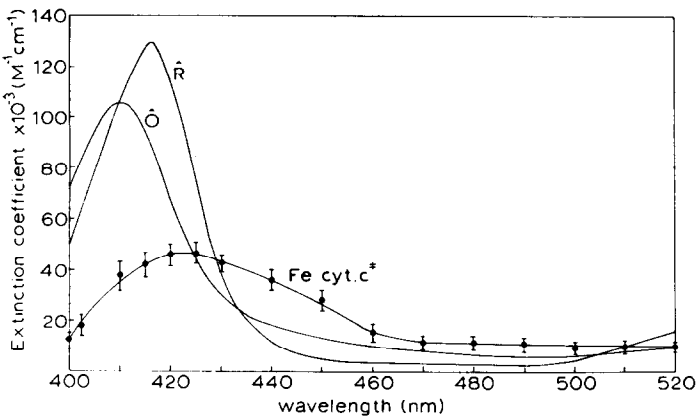

Fig. 3. The absorption spectrum of the intermediate (Fe cyt. $c^{\neq}$) at pH 7.4 calculated as in the text. For comparison, the spectrum of oxidised cytochrome c ($\hat{O}$) and reduced cytochrome c ($\hat{R}$) are included.

spectrum of the intermediate in the 400–520 nm range, derived in this manner, is shown in Fig. 3. As mentioned above, the spectrum in the 520–610 nm range is similar to that of ferricytochrome c.

The development of the absorbance at 606 nm

Van Buuren *et al.* [3] have described the spectral changes that occur when ferricytochrome c is allowed to react with an excess of hydrated electrons. Besides small shifts in the position of the α- and β-peaks of ferrocytochrome c a most conspicuous change was the formation of an absorption band at 606 nm. The ferric species produced in step II of the present pulse-radiolysis experiments was, however, found to develop no measurable peak in absorbance in the range 600–610 nm up to a period of 2 min after the pulse. It therefore seems possible that the 606 nm absorption band is very slowly formed.

Anaerobic solutions of ferrocytochrome c were irradiated with γ-rays from a ^{60}Co source for a few minutes so that an approximate stoichiometric amount of hydrated electrons was generated. When the spectrum was measured immediately after irradiation, a shift of a few nm in the position of the α-peak of ferrocytochrome c to higher wavelength and of the β-peak to lower wavelength was observed, similar to that described in ref. 3. However, the full development of the species with an absorption band at 606 nm was not observed until 30 min after the end of the irradiation (*cf.* Fig. 5).

It has been shown that when HS^- ions react with myoglobin in the presence of H_2O_2, a sulfmyoglobin is formed which has an absorption maximum at around 617 nm [12, 13]. Although the formation of the present compound with a peak at 606 nm is not affected by the addition of catalase after the irradiation, showing that H_2O_2 produced by the irradiation is not required in the reaction, it seems nevertheless possible that a similar type of product is formed. The combined reducing equivalents of the ferrohaem and the aqueous electron might give rise to reduction of one of the thioether bonds linking the haem to the protein and thus produce a sulfhydryl group.

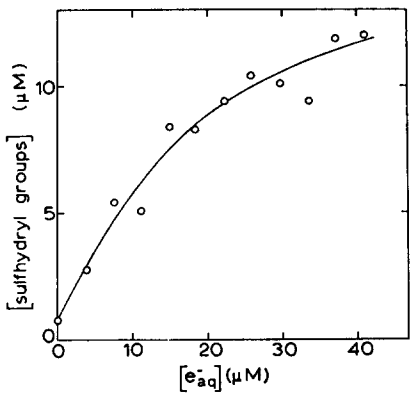

Fig. 4. Determination of sulfhydryl groups formed by reaction of ferrocytochrome *c* with varying amounts of hydrated electrons. An anaerobic solution of 40 μM ferrocytochrome *c* was irradiated for varying periods of time with a ^{60}Co source at a dose-rate of 2600 rad/min. The solution contained 2 mM phosphate buffer (pH 7.4) and 1 M *tert*-butanol. Immediately after the irradiation was completed sulfhydryl groups were determined spectrophotometrically with PCMB [14]. One aliquot of 2.4ml was added to 0.6 ml of a mixture containing 125 mM phosphate buffer (pH 7.0), 2.5 % sodium dodecyl sulfate and 200 μM PCMB. Another aliquot was diluted with the same solution minus PCMB. From the difference in absorbance at 250 nm between these solutions and after subtraction of the absorbance of the PCMB alone, the concentration of sulfhydryl groups was assayed, using $\Delta\varepsilon^{250\,nm} = 13\ mM^{-1} \cdot cm^{-1}$ (determined by titration).

We therefore decided to investigate whether sulfhydryl groups are formed by reaction with e_{aq}^- and whether they are a cause of the development of the absorption band at 606 nm.

An anaerobic solution of 40 μM ferrocytochrome *c* was irradiated with a ^{60}Co source (Gammacell 200, Atomic Energy of Canada) for varying periods of time at an intensity of 2600 rad/min, generating 7.4 μM e_{aq}^- per min. Sulfhydryl groups were then immediately determined spectrophotometrically using PCMB [14]. 0.5 % sodium

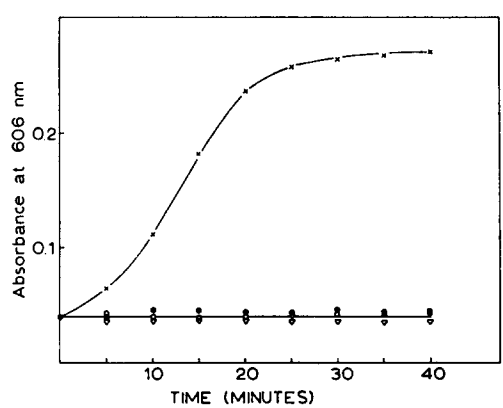

Fig. 5. The development of the 606 nm absorption in a 1-cm cell of a solution of ferricytochrome *c* ($36 \cdot 10^{-6}$ M) and 1.0 M *tert*-butanol at pH 7.4 which had been irradiated in a ^{60}Co source for 15 min (e_{aq}^-/cytochrome *c* = 3.1). Additions: ●, 100 μM 5,5′-dithio-bis(2-nitrobenzoic acid); ○, 100 μM *p*-chloromercuribenzoic acid; ▽, 4 M urea; and ×, no addition.

dodecyl sulfate was present in the assay mixture to convert all ferrocytochrome *c* to the ferric form by autoxidation. It can be seen from Fig. 4 that sulfhydryl groups are formed with an initial stoichiometry of approx. 0.6 mol sulfhydryl formed per mol e_{aq}^-.

The involvement of the sulfhydryl groups in the formation of the product with an absorption band at 606 nm is indicated by the following experiment. An anaerobic solution of ferrocytochrome *c* was irradiated with the ^{60}Co source so that the total amount of hydrated electrons produced in the solutions was 3.0 times that of the cytochrome. Immediately after irradiation 1.2-ml samples were added to 1-cm cuvettes which contained 25 mM potassium phosphate buffer (final concentration) and sulfhydryl reagents or urea such that the final volume of the solutions in the cuvettes were 2.7 ml. The absorbance at 606 nm was then recorded every 5 min. A typical result is shown in Fig. 5. It can be seen from the figure that the development of the absorbance at 606 nm is completely prevented by the sulfhydryl reagents and also by the presence of 4 M urea which unfolds the protein. This experiment suggests that the sulfhydryl groups, when in a specific configuration, are responsible for the development of the absorption band at 606 nm. This is further indicated by the fact that after full development of this absorption band sulfhydryl groups are no longer detectable (not shown).

DISCUSSION

The reaction of hydrated electrons with ferrocytochrome *c* is similar to that of the ferricytochrome *c* reaction [10] in that in both cases the rates of the reactions are dependent on a p*K* of the cytochromes at around 9.0. With ferricytochrome *c* it is believed that the change in activity is due to the replacement of the methionine-80 ligand by a lysine ligand [15] possibly brought about by the deprotonation of the lysine side chain at higher pH values. However as methionine-80 remains the ligand in ferrocytochrome *c* up to at least pH 12.0 [16], it may be possible that the state of protonation of the lysine is very relevant to the activity of the two forms of the cytochrome.

The intermediate which is formed as a product of step I has a broad absorption in the Soret region (Fig. 3). This absorption is quite different to the sharp intense Soret peaks of the normal ferro- or ferrihaemoproteins. It is therefore presumed that this low broad absorption is due to the presence of a ferroporphyrin anion radical in the cytochrome [17]. Ferroporphyrin anions have been previously formed by the reduction of ferroporphyrins by strong reducing agents ($E_0 < -1.0$ V), *e.g.* sodium amalgam [18] or polarography [19]. The relatively high yield of this species (0.7 $[e_{aq}^-]_o$) shows that the haem of the ferrocytochrome *c* is apparently almost as efficient as ferricytochrome *c* [4, 20] in receiving the charge from the aqueous electron. Preliminary experiments with demetallized cytochrome *c* (de Kok, J., unpublished) have also shown that with hydrated electrons a porphyrin anion radical is produced. It is therefore suggested that the porphyrin in cytochrome *c* is the main attractant for electrons.

The similarity of the spectrum in the range 400–610 nm of the product at 50 μs to that of the normal ferricytochrome *c* is taken to imply that this product is a form of ferricytochrome *c*. This species is presumably formed via the donation of two reducing

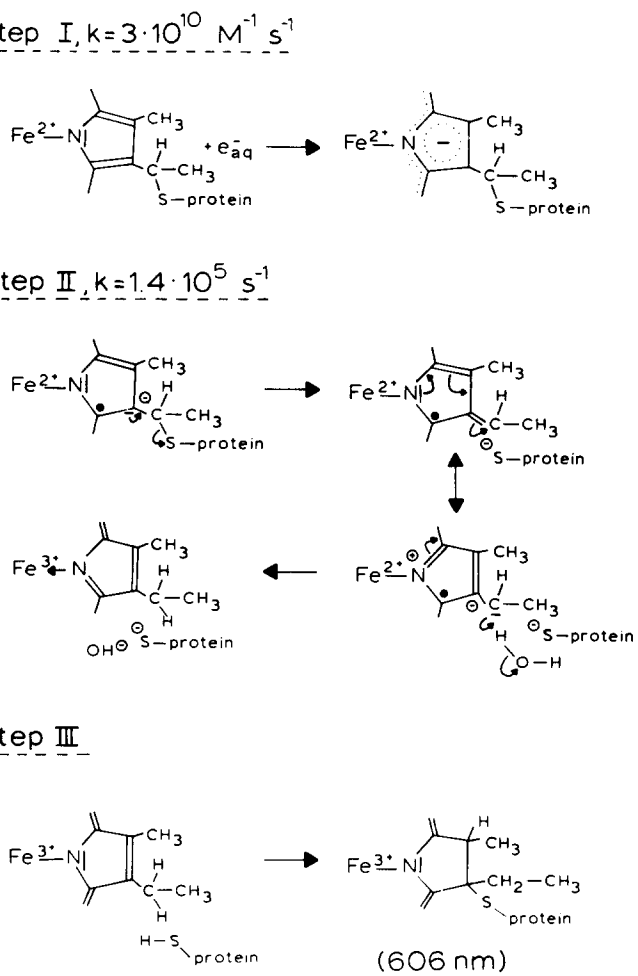

Fig. 6. A mechanism for the reactions that lead to the formation of a compound with an absorption peak at 606 nm and a hypothetical structure of this compound.

equivalents contained in the ferroporphyrin anion radical to a group outside the porphyrin ring. The amount of ferric species formed per hydrated electron in the pulse-radiolysis experiments is approximately equal to the amount of sulfhydryl formed per hydrated electron produced by low doses of γ-rays from a ^{60}Co source. This indicates that the two reducing equivalents of the ferroporphyrin anion radical are used to reduce and hence cleave a thioether bond which links the haem to the cysteine (14 or 17). It is presumably this product that shows, in the reduced form, shifts of a few nm in the position of the α- and β-peaks compared to native ferrocytochrome *c*. A mechanism for the internal oxidation-reduction process is proposed in Fig. 6, step II.

This explanation stresses the feasibility of oxidation-reduction mechanisms for cytochrome *c* that involve electron transfer to and from the iron via the porphyrin ring [21, 22].

It is suggested in Fig. 5, step III that the final product with an absorption band at 606 nm has a modified porphyrin ring produced by the attack of a sulfhydryl group, and that the structure is analogous to that proposed by Nichol *et al.* [13] for sulfmyoglobin.

ACKNOWLEDGEMENTS

Two of us (J. B. and J. de K.) wish to thank the University of Utrecht for a short-term fellowship. This research has been supported in part by the Netherlands Organization for the Advancement of Pure Research (Z.W.O.) under the auspices of the Foundation for Biophysics.

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