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THE REDUCTION OF PORPHYRIN CYTOCHROME *c* BY HYDRATED ELECTRONS AND THE SUBSEQUENT ELECTRON TRANSFER REACTION FROM REDUCED PORPHYRIN CYTOCHROME *c* TO FERRICYTOCHROME *c*

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SUMMARY

1. Hydrated electrons, produced by pulse radiolysis, react with porphyrin cytochrome *c* with a bimolecular rate constant of $3 \cdot 10^{10}$ $M^{-1} \cdot s^{-1}$ at 21 °C and pH 7.4.

2. After the reduction step an absorbance change with a half-life of 5 μs is observed in the spectral range of 430–470 nm. A relatively stable intermediate then decays with a half-life of 15 s.

3. The spectrum of the intermediate observed 50 μs after the generation of hydrated electrons shows a broad absorption band between 600 and 700 nm and a peak at 408 nm. The spectrum is attributed to the protonated form of an initially produced porphyrin anion radical.

4. Reduced porphyrin cytochrome *c* reacts with ferricytochrome *c* with a bimolecular rate constant of $2 \cdot 10^5$ $M^{-1} \cdot s^{-1}$ in 2 mM phosphate pH 7.4, at 21 °C and of $2 \cdot 10^6$ $M^{-1} \cdot s^{-1}$ under the same conditions but at 1 M ionic strength. It is proposed that electron transfer in the analogous exchange reaction between ferrocytochrome *c* and ferricytochrome *c* occurs via the exposed part of the haem.

INTRODUCTION

Hydrated electrons are capable of reducing ferricytochrome *c* extremely rapidly ($k = 10^{10}$–10^{11} $M^{-1} \cdot s^{-1}$, for review cf. ref. 1). With the pulse radiolysis technique it is possible to produce hydrated electrons within a microsecond and to follow the subsequent absorbance changes spectrophotometrically. The technique enabled Van Buuren et al. [2] to follow with high time-resolution the electron-transfer reaction from ferrocytochrome *c*, after its rapid formation from ferricytochrome *c* by hydrated electrons, to cytochrome *c* oxidase.

The effect of chemical modification of the protein moiety of cytochrome *c* on its electron-transfer function has been extensively studied (for review cf. ref. 3). It

would be interesting to know in how far the specificity of the electron-transfer reactions of cytochrome *c* is determined by the electronic configuration of the prosthetic group. Recently, it has been found that cytochrome *c*, in which the iron atom had been replaced by cobalt, showed good activity with cytochrome *c* oxidase [4]. It might also be useful to investigate whether electron transfer is possible solely on basis of the redox properties of the porphyrin ring of cytochrome *c*, i.e. in the absence of a redox-active central metal ion.

Porphyrins have been electrochemically reduced in aprotic media to porphyrin anion radicals at potentials of around −1.0 to −1.5 V vs. the saturated calomel electrode [5, 6]. Reduction of porphyrins has also been effected in aqueous media with the help of hydrated electrons and other radicals produced by pulse radiolysis [7].

Iron can be removed from cytochrome *c* to form porphyrin cytochrome *c* [8] without significantly affecting the protein structure of the enzyme [8–10]. This encouraged us to investigate whether the hydrated electron reacts with porphyrin cytochrome *c* to generate a porphyrin radical and whether this radical transfers electrons to haemoproteins.

MATERIALS AND METHODS

Materials

Horse heart cytochrome *c* was obtained as Type VI from Sigma. Anhydrous hydrogen fluoride was obtained from Matheson; possible traces of water were removed with CoF_3. *t*-Butanol was purissima grade from Fluka. All other chemicals were of A.R. grade.

Preparation of porphyrin cytochrome c

Porphyrin cytochrome *c* was prepared by treatment of 200 mg lyophilized cytochrome *c* with anhydrous hydrogen fluoride using a similar method and apparatus as described by Flatmark and Robinson [8]. The product was purified by chromatography on Sephadex G-50 fine and Amberlite CG 50 [9, 10] and was obtained in 40% yield. The concentration was calculated on basis of an extinction coefficient of 13.0 $mM^{-1} \cdot cm^{-1}$ at 504 nm [8]. All handling of porphyrin cytochrome *c* was performed under conditions that minimized light exposure.

Pulse radiolysis

Hydrated electrons were generated by delivering a 0.5 μs pulse of 2 MeV electrons from a Van de Graaff accelerator to cytochrome *c* preparations dissolved in a matrix solution. This solution consisted of anaerobic, argon-saturated 2 mM phosphate buffer (pH 7.4) in triple-distilled water to which 100 mM t-butanol was added to scavenge $OH^{\cdot}$ radicals. All reactions were carried out at 21 °C and were followed by fast spectrometry essentially as described previously [2]. At higher wavelengths a CVP 150 photomultiplier was used.

The concentration of hydrated electrons was determined by using an extinction coefficient of 14.1 $mM^{-1} \cdot cm^{-1}$ at 650 nm [11]. Cytochrome *c* concentrations were calculated on basis of an extinction coefficient of 21.1 $mM^{-1} \cdot cm^{-1}$ (red minus ox) at 550 nm [12].

The total amount of hydrated electrons generated per pulse was determined

by a charge-collecting method which was calibrated by measuring the absorbance at 650 nm immediately after a pulse in a matrix solution where the lifetime of the hydrated electron is much longer than the pulse length.

The concentration of hydrated electrons generated in solutions of porphyrin cytochrome *c* was kept below 10 % of the concentration of the protein in order to minimize two-equivalent reduction of the latter.

RESULTS

The reaction of hydrated electrons with porphyrin cytochrome *c* (10–30 μM) was followed at various wavelengths. Porphyrin cytochrome *c* was present in a more than tenfold excess so that the reaction obeyed pseudo-first-order kinetics. From the absorbance changes at 650 nm, the second-order rate constant for the reaction of hydrated electrons with porphyrin cytochrome *c* was calculated to be $3 \pm 0.5 \cdot 10^{10}$ $M^{-1} \cdot s^{-1}$ (21 °C, 2 mM phosphate, pH 7.4). Fig. 1A shows the sudden decrease in transmittance at 650 nm due to the generation of hydrated electrons and the subsequent increase caused by their reaction with porphyrin cytochrome *c*. The transmittance does not completely return to the initial level showing that the porphyrin cytochrome *c* product has a higher absorbance at 650 nm than the parent substance. The rate of the reaction is independent of wavelength (400–800 nm) and thus the rate of the disappearance of hydrated electrons and that of the formation of porphyrin cytochrome *c* product is the same.

The absorbance of the porphyrin cytochrome *c* product attained after completion of the reaction with hydrated electrons is relatively stable ($t_{\frac{1}{2}} = 15$ s) over the whole spectral range investigated except at 430–470 nm where the attained absorbance decays with a half-life of 5 μs to a slightly lower absorbance. This is illustrated in Fig. 1B where the transmittance changes at 450 nm are shown. At this wavelength the extent of the absorbance change with a half-life of 5 μs is maximal. After this process, the absorbance at 430–470 nm changes at the same rate as outside this spectral range.

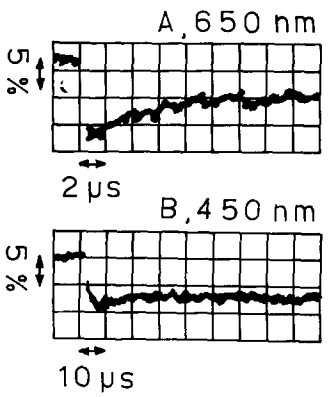

Fig. 1. Transmittance changes due to the generation of hydrated electrons in a solution of porphyrin cytochrome *c*. A, 650 nm, 1.1 μM hydrated electrons; B, 450 nm, 1.2 μM hydrated electrons. The solution contained 12.4 μM porphyrin cytochrome *c*. For further conditions see Methods.

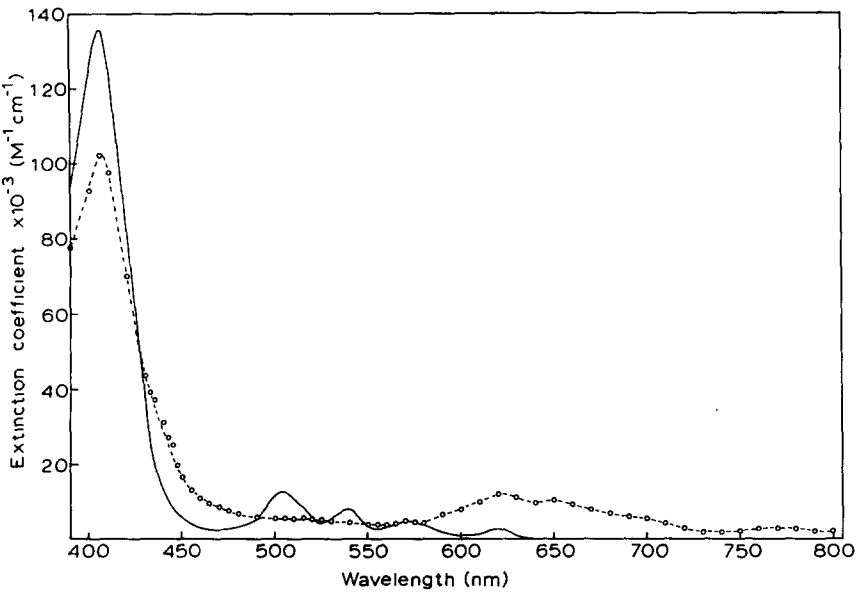

Fig. 2. Absolute absorption spectra of porphyrin cytochrome *c* (—) and the intermediate derived from it by single-electron reduction (o--o). Absorbance changes were measured 50 μs after generation of hydrated electrons in solutions containing a more than tenfold excess of porphyrin cytochrome *c* (5–30 μM). The absolute spectrum of the intermediate was calculated assuming that 65 % of the hydrated electrons reacted with the porphyrin moiety (cf. text).

Fig. 2 shows the absolute absorption spectrum of porphyrin cytochrome *c* and that of the relatively stable intermediate measured 50 μs after the pulse. The spectrum of the intermediate is similar to that of porphyrin anion radicals in an aprotic solvent, in particular as far as the absorbance in the region of 600–700 nm is concerned [5]. The main difference is that a single large absorption band is found in the 400–450 nm region whereas the spectrum of known anion radicals in an aprotic solvent shows in addition to a main peak at around 400 nm a second, smaller, peak at around 440 nm. It is conceivable that the initially attained absorbance in the range of 430–470 nm which decays with a half-life of 5 μs (see above) corresponds to the latter peak. A reasonable explanation is that the absorbance changes at these wavelengths are due to protonation of an initially formed anion radical. The short half-life of this reaction and the fact that the rate constant is not increased by lowering the pH to 6.0 indicate that a proton is abstracted from water. The suggestion that the change with a half-life of 5 μs is due to protonation could be in accordance with the proposal that the anion radical of free haematoporphyrin in water becomes protonated in less than a microsecond [7]. It is interesting to note that in the spectrum of the free haematoporphyrin radical in water an absorption band at 440 nm is also hardly distinguishable [7].

The relatively stable intermediate assigned to a protonated anion radical is converted in a first-order mode with a half-life of 15 ± 5 s (cf. Fig. 3A). The half-life of this decay was found to be independent of the concentrations of porphyrin cytochrome *c* and hydrated electrons.

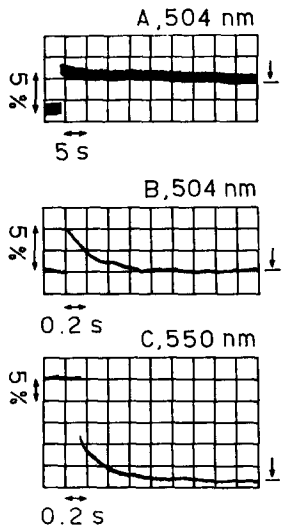

Fig. 3. Transmittance changes after the generation of hydrated electrons in a solution of porphyrin cytochrome *c* (A) and in a mixture of porphyrin cytochrome *c* and ferricytochrome *c* (B and C). A, 504 nm, 20 μM porphyrin cytochrome *c*, 1.9 μM hydrated electrons; B, 550 nm, 20 μM porphyrin cytochrome *c*, 22 μM ferricytochrome *c*, 3.1 μM hydrated electrons; C, 504 nm, 20 μM porphyrin cytochrome *c*, 22 μM ferricytochrome *c*, 3.1 μM hydrated electrons. For further conditions see Methods.

The different steps that can be discerned when the hydrated electron reacts with porphyrin cytochrome *c* are summarized in Table I.

Only a part of the number of generated electrons may react with the porphyrin ring, the remaining part being lost to the protein moiety. The fraction of hydrated electrons that produce the porphyrin radical, defined as the yield, was estimated by measuring the amount of ferrocytochrome *c* that can be formed from ferricytochrome *c* by reaction with reduced porphyrin cytochrome *c*.

Fig. 3 shows transmittance changes at 504 and 550 nm occurring after the generation of hydrated electrons in a solution of porphyrin cytochrome *c* (Trace A) and in a mixture of porphyrin cytochrome *c* and ferricytochrome *c* (Traces B and C). These wavelengths were selected for measurement, since at 504 nm a maximal absorbance change is observed for the reduction of porphyrin cytochrome *c* and in

TABLE I

REACTIONS OCCURRING UPON GENERATION OF HYDRATED ELECTRONS IN A SOLUTION OF PORPHYRIN CYTOCHROME *c*

For conditions see Methods. $k_1 = 3 \cdot 10^{10}\ M^{-1} \cdot s^{-1}$ (pH 7.4); $k_2 = 1.4 \cdot 10^5\ s^{-1}$; $k_3 = 4.6 \cdot 10^{-2}\ s^{-1}$.

$$e^-_{aq} + PH_2 \text{ cyt. } c \xrightarrow{k_1} PH_2^- \text{ cyt. } c \xrightarrow[H_2O]{k_2} PH_3^{\cdot} \text{ cyt. } c + OH^-$$

$$\downarrow k_3$$

final product(s)

addition this wavelength is isosbestic for redox changes of cytochrome *c* while at 550 nm reduced cytochrome *c* has the absorbance maximum of its α-band and in addition this wavelength is nearly isosbestic for the reduction of porphyrin cytochrome *c*. When hydrated electrons are generated in a mixture of the two species of cytochrome *c*, the transmittance at 504 nm observed instantaneously after the pulse returns in 1 s to its original level (Trace B), indicating that the porphyrin radical transfers its electron to ferricytochrome *c*. This is consistent with the observation at 550 nm where the change in transmittance is biphasic (Trace C). The initial, rapid change at 550 nm can be ascribed to the reduction of ferricytochrome *c* by a portion of the hydrated electrons, whereas the slower, secondary change occurs at the same rate as the transmittance change at 504 nm. Therefore, this second reaction is attributed to the reduction of ferricytochrome *c* by the porphyrin radical.

The amount of reducing equivalents, corresponding to that of the hydrated electrons that have reacted with the protein part of porphyrin cytochrome *c*, is assumed not to be transferable to ferricytochrome *c*. This amount is determined from the difference in total absorbance change at 550 nm produced by a certain amount of hydrated electrons in a pure ferricytochrome *c* solution and that in a mixture of porphyrin cytochrome *c* and ferricytochrome *c* (Trace C). On this basis the yield of the reaction of hydrated electrons with porphyrin cytochrome *c* was calculated to be $65 \pm 5\,\%$. In this calculation use has been made of the finding that the yield of the reaction with ferricytochrome *c* was virtually 100 %. However, these yields may be overestimated by approx. 10 % due to a contribution of hydrogen atoms to the reduction reaction [1].

Fig. 4 shows a semilogarithmic plot of the reaction of the porphyrin radical with ferricytochrome *c*, derived from the secondary absorbance changes measured at 550 nm (cf. Fig. 3C). The reaction is pseudo-first-order in ferricytochrome *c*. In 2 mM phosphate the second-order rate constant is calculated to be $2 \pm 0.2 \cdot 10^5$ $M^{-1} \cdot s^{-1}$. However, under the same conditions but at higher ionic strength (2 mM phosphate pH 7.4 and 1 M $KClO_4$) the rate constant ($2 \pm 0.5 \cdot 10^6$ $M^{-1} \cdot s^{-1}$) was found to be one order of magnitude higher than at low ionic strength.

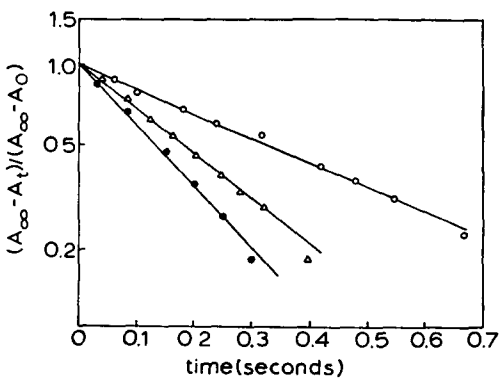

Fig. 4. The reduction of ferricytochrome *c* by porphyrin cytochrome *c* radicals calculated from the absorbance changes at 550 nm (cf. Fig. 2C). A_0 = absorbance at the start of the secondary phase; A_∞ = absorbance at infinite time, ○—○, 9.8 μM cytochrome *c*; (▲—▲), 22.2 μM cytochrome *c*; ●—●, 30.1 μM cytochrome *c*. All solutions also contained 20 μM porphyrin cytochrome *c*. For further conditions see Methods.

DISCUSSION

Recently we found that the hydrated electron reacts with ferrocytochrome *c* with a rate constant of $3 \cdot 10^{10}$ $M^{-1} \cdot s^{-1}$ and with a yield of 70 % [13]. These values are the same as those of the reaction with porphyrin cytochrome *c*, which was carried out under comparable conditions. We have proposed that it is the porphyrin ring of ferrocytochrome *c* which is reduced by the hydrated electron. The product formed is, however, unstable and decays with a half-life of 5 μs to form a species of ferricytochrome *c*. The formation of this species was attributed to the transfer of two reducing equivalents, obtained from the ferro ion and the additional electron on the porphyrin ring, to one of the thioether bonds that link the haem to the polypeptide chain. The intermediate formed by the one-equivalent reduction of porphyrin cytochrome *c* appears to be much more stable and decays with a half-life of 15 s. The intermediate is attributed to a protonated radical.

The porphyrin anion radical of free porphyrin was found to disproportionate in water to a phlorin and the parent porphyrin [5, 7]. However, the spectral changes observed after the generation of hydrated electrons by γ-irradiation in solutions of porphyrin cytochrome *c* did not show the formation of a band in the near-infrared, which would have been indicative for the formation of a phlorin [5, 7]. The spectra suggest that the final product(s) show(s) four visible bands but with lower intensities than the parent substance.

The comparatively high stability of the porphyrin radical has permitted the measurement of the electron-transfer reaction of the latter compound to ferricytochrome *c*. It is unlikely that in this electron-transfer reaction the electron leaves the porphyrin ring of porphyrin cytochrome *c* via the residues which function in native cytochrome *c* as axial ligands. This suggests then that in the analogous electron-exchange reaction between ferrocytochrome *c* and ferricytochrome *c* the electrons can be transferred via the exposed part of the haem, in accordance with earlier proposals [3, 14, 15].

Gupta [16] measured a rate constant for the electron-exchange reaction between ferrocytochrome *c* and ferricytochrome *c* of $1 \cdot 10^4$ $M^{-1} \cdot s^{-1}$ at 1 M ionic strength, neutral pH and 40 °C. The rate constant of the electron-transfer reaction of the porphyrin radical to ferricytochrome *c* was found to be $2 \cdot 10^6$ $M^{-1} \cdot s^{-1}$ under comparable conditions but at 21 °C. This rate constant is thus at least a factor 10^2 higher than that of the electron-exchange reaction. This difference in rate constants can be qualitatively explained on basis of Marcus' theory [15, 17, 18], since the reduction potential at pH 7 of the porphyrin cytochrome *c*/porphyrin cytochrome *c* radical couple is estimated to be more than 1 V lower than that of the ferricytochrome *c*/ferrocytochrome *c* couple. A high rate of the reaction under concern is then predicted, provided that the rate of the electron-exchange reaction between reduced porphyrin cytochrome *c* and porphyrin cytochrome *c* is not extremely slow. Electron-exchange reactions of aromatic molecules with their anion radicals are, however, usually quite rapid (cf. e.g. refs. 19, 20). A factor which may also contribute to the relatively high rate of the reaction under concern is the fact that the electron is removed from a porphyrin π^* orbital which may extend far into the solvent and thus give a high degree of overlap with the pertinent ferrihaem orbitals of ferricytochrome *c*. This would be particularly important in the case of cytochrome *c* where the por-

phyrin ring is located in a crevice [15].

The hydrated electron reduces the porphyrin rings of porphyrin cytochrome *c* and ferrocytochrome *c* [13] and the ferrihaem of ferricytochrome *c* with similar rate constants of $3\text{–}5 \cdot 10^{10}$ $M^{-1} \cdot s^{-1}$ under conditions as employed here [1, 21]. These rates are near the diffusion-controlled limit [21]. The correspondence in rate suggests that these reactions may occur via a similar mechanism, involving a similar type of 'redox orbitals'. In view of the present work these might be the π^* orbitals of the porphyrin ring. It was proposed [22] that the high rate constants of many reduction reactions involving hydrated electrons are due, in part, to the low redox potential of this species ($E_0 = -2.9$ V, ref. 23). This would enable hydrated electrons to enter transition-metal complexes via vacant orbitals that are energetically not accessible to most other reducing agents. The fact that the hydrated electron reacts with ferricytochrome *c* at a nearly diffusion-controlled rate might thus be partly ascribed to the energetic and steric accessibility of the porphyrin π^* orbitals.

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