Electron Transfer to Ferricytochrome c: Reaction with Hydrated Electrons and Conformational Transitions Involved

(horse heart cytochrome c/pulse radiolysis/*Pseudomonas* cytochrome c/protein relaxation/difference spectra)

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ABSTRACT The reaction of horse-heart cytochrome c with hydrated electrons has been studied by the pulse-radiolysis technique. In neutral solution, the ferriheme group was reduced in a bimolecular reaction that takes place at a rate equal to that of the decay of the e^-_{aq}, and approaches the diffusion-controlled limit. This reduction is assigned mainly to a direct reaction, proceeding via the exposed edge of the porphyrin projecting into the cytochrome c crevice.

The reaction absorption spectrum observed 20 μsec after an electron pulse was very similar, yet blue-shifted relative to, the difference spectrum between the reduced and oxidized forms of cytochrome c. However, this shift vanishes in a slow monomolecular reaction, which seems to reflect the conformational relaxation of the protein to the final equilibrium state of its reduced form. In alkaline solutions, the transition of cytochrome c molecules into an irreducible conformation causes a proportionate decrease in the amount of ferricytochrome c reduced in the direct reaction. The rate of conformational transition of the protein into the reactive form is now the limiting step for a substantial part of the reduction that takes place via this slow monomolecular reaction.

Pseudomonas cytochrome c 551 which, in contrast to horse-heart cytochrome c, is a negatively charged protein at neutral pH reacts with e^-_{aq} at a rate lower than does the horse-heart protein. The reduction of the heme group follows that of the e^-_{aq} decay with a small, yet significant, delay.

Cytochrome c is found in the mitochondria of all aerobic organisms. It is a heme protein that is built of a single polypeptide chain, with a covalently bound heme group. The redox function of the protein is accomplished by its shuttling between the ferri and ferro oxidation states of the heme-bound iron, thus transferring single electrons from cytochrome c reductase to cytochrome oxidase (1). The three-dimensional structure of both horse-heart ferri- and tuna-heart ferro-cytochrome c has been determined to 2.8- and 2.45-Å resolution, respectively (2, 3). Their structural analyses provided a detailed picture of the heme group embedded within the protein, and of the considerable conformational differences between the two oxidation states (3). The detailed mechanism of the electron-transfer process mediated by this protein still awaits elucidation. Although the electron-transfer mechanism of heme proteins has been a subject of much concern and discussion (4, 5), the experimental evidence gathered in support of any of the suggested mechanisms is rather limited (15). Thus, it became desirable to try

to correlate the detailed structural data on cytochrome c with the elementary steps involved in its reduction. Dickerson *et al.* have recently proposed a mechanism for the electron transfer to cytochrome c (6). This mechanism was based on the transient free-radical model originated by Winfield (7), and is compatible with the information obtained by both chemical modification and structural studies (6).

The technique of pulse radiolysis is very useful in tracing the elementary steps involved in redox reactions (8, 9). Typical examples are the identification of unstable intermediate oxidation states of transition-metal ions (10) or semiquinonoid forms of biochemically important prosthetic groups (11, 12). The technique is based on the perturbation of the examined spectrum by a short pulse ($\leqslant 1$ μsec) of high-energy radiation (8). These pulses interact with water to produce reactive radicals ($H_2O \rightarrow e^-_{aq}, OH, H$). Prominent among these products is the hydrated electron, which is an elementary and most-potent reducing agent in aqueous solutions. The transient species produced by the radiation pulse and chemical events induced by it may be followed on an appropriate spectrophotometer (8, 9). By the addition of specific scavengers, it is possible to eliminate all other products except those to be studied.

In a recent preliminary communication, the reduction of horse-heart ferricytochrome c by hydrated electrons was shown to be a direct bimolecular process of a specific rate, which approached very closely the diffusion-controlled limit (13). This reduction process has now been further pursued, and details of the dynamics of the conformational transition involved in the reduction have been resolved. The reactivity of P-551 cytochrome c from *Pseudomonas fluorescens* towards the hydrated electron has also been investigated. This cytochrome c differs from its eukaryotic homologs in its total number, composition, and order of residues in the amino-acid sequence (1, 14). P-551 cytochrome c is also reduced by an e^-_{aq} in a bimolecular reaction, which is somewhat slower than for horse-heart cytochrome c. This behavior is expected in view of the net negative charge of the *Pseudomonas* protein.

EXPERIMENTAL PROCEDURE

Materials. Horse-heart cytochrome c was supplied by Sigma Chemical Co., St. Louis, Mo. Only protein prepared by acetic-acid extraction (type VI) was used. Traces of the reduced form were reoxidized by ferricyanide, followed by further purification on an Amberlite CG 50 column.

P-551 cytochrome c was prepared from an acetone powder

Abbreviation: e^-_{aq}, hydrated electron.

of *Pseudomonas fluorescens* by the procedure described by Ambler (14).

Tertiary butanol was G. R. Grade of Merck, A. G. Darmstadt. All other chemicals were of analytical grade and were used without further purification.

Methods. All solutions were prepared in triple-distilled water. Oxygen was removed from the water by continuous bubbling (≥ 30 min) of highly purified argon (gas chromatographic analysis has proven that $[O_2] \leq 0.1$ μM). No buffers were used for the following reasons: (*a*) previous studies have shown that ion binding affects the reactivity of cytochrome *c* (1, 13, 15, 16) and (*b*) both binding buffers (phosphate) and nonbinding buffers (cacodylate) interfere by reacting with the hydrated electrons; thus, the pH of the solutions was adjusted by the addition of dilute base or acid. All solutions contained 0.1 M tertiary butanol, which completely and specifically scavenged OH radicals by converting them into relatively unreactive $HOCH_2C(CH_3)_2$ radicals (17). Different cytochrome *c* concentrations (0.5–40 μM) were prepared by addition of appropriate volumes of freshly prepared concentrated stock solutions. All measurements were done at 20°.

The pulse-radiolysis system of the Hebrew University of Jerusalem was used. A Varian linear accelerator operating at 5 MeV and 200 mA was used as the electron-pulse source. The dose was varied by modulating the pulse duration between 0.1 and 1.0 μsec, to give a dose range of 150–3000 rads. The electron pulse intensity varied within $\pm 5\%$. The inductive current obtained in a coil by the passage of the electron pulse was used to monitor its intensity. A rectangular flow-through, multiple reflection cell ($1 \times 2 \times 4$ cm) with a light path of 12.4 cm (3 passes) was used. The cell was made of high-purity quartz in order to minimize artifacts from radiation-induced fluorescence. The electron beam was perpendicular to the incident analyzing light, and passed through 1 cm of the solution. The anaerobic cell-filling method was based on that originally designed by Christiansen *et al.* (18).

An Osram 150 W xenon arc was used as the light source. The transmitted light intensities were analyzed and monitored by a system composed of a Bausch and Lomb high-intensity monochromator, a 1P28A photomultiplier, and a Tektronix 556 double-beam oscilloscope fitted with a Polaroid camera. Transmittance changes were recorded by photographing the oscilloscope traces. Light filters were used in order to avoid second-order light signals, as well as to eliminate possible photolytic effects in the examined solutions. A shutter was used between the irradiation cell and the light
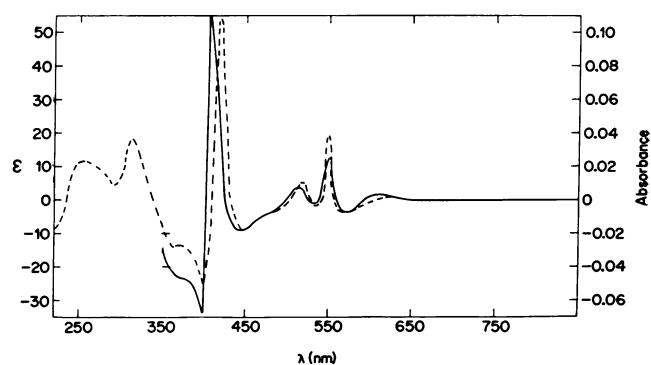

FIG. 1. Transient and difference spectra of cytochrome *c*. *Right scale:* absorption changes recorded 20 μsec after the pulse; [cytochrome *c*] = 1.0×10^{-5} M, dose = 200 rads. *Left scale:* (– – –) difference spectra (reduced–oxidized) of cytochrome *c* according to Margoliash and Frohwirt (19).

source in order to protect the photomultiplier and the solutions from excessive illumination.

A manual trace follower coupled to an analog to digital converter was used to read the traces on the Polaroid pictures and to transfer the data to punch cards, which were then used in an appropriate computer program to analyze the data. All traces were analyzed for the total change of absorbance and for their correlation with either first- or second-order decay. Pseudo first- or second-order constants were calculated when satisfactory decay plots were obtained for at least two half-lives. At least five traces were analyzed for every rate constant or point in the spectrum.

RESULTS

Neutral solutions of horse-heart cytochrome *c* (pH 6–7)

After the electron pulse, fast transmittance changes were observed over the whole wavelength range studied, namely, from 350 to 750 nm. In the region between 600 and 750 nm, these changes were mainly due to the appearance and decay of hydrated electrons, which have a very broad absorption band centered at 720 nm (9, 13). The decay of the e^-_{aq} band is a bimolecular process, having the rates shown in Table 1.

At wavelengths below 600 nm, transmittance changes were due mainly to the changes of the cytochrome *c* absorbance (13). From analysis of the decay curves of the e^-_{aq} and ferricytochrome *c*, it again became evident that the two processes are synchronous (Table 1).

The amplitudes of the absorbance changes were measured 20 μsec after the pulse (between 350 and 750 nm), and are plotted in Fig. 1. The difference spectrum between the reduced and oxidized formes of cytochrome *c* was drawn according to the data of Margoliash and Frohwirt (19), and is also shown in Fig. 1. The shift between the two spectra is significant, especially in the Soret region. The spectra of the absorbance changes 1 sec and 50 sec after the pulses were recorded over the same wavelength region and were found to be identical to the spectrum measured by Margoliash and Frohwirt (Fig. 1). Thus, the spectral shift disappears, and the reaction spectra observed at longer periods of time after the pulse are identical with the static difference spectrum between the two oxidation states of the protein. This change of the spectrum produced 20 μsec after the pulse to the final equilibrium form proceeded in a first-order rate of 8.5 ± 0.3 sec^{-1},
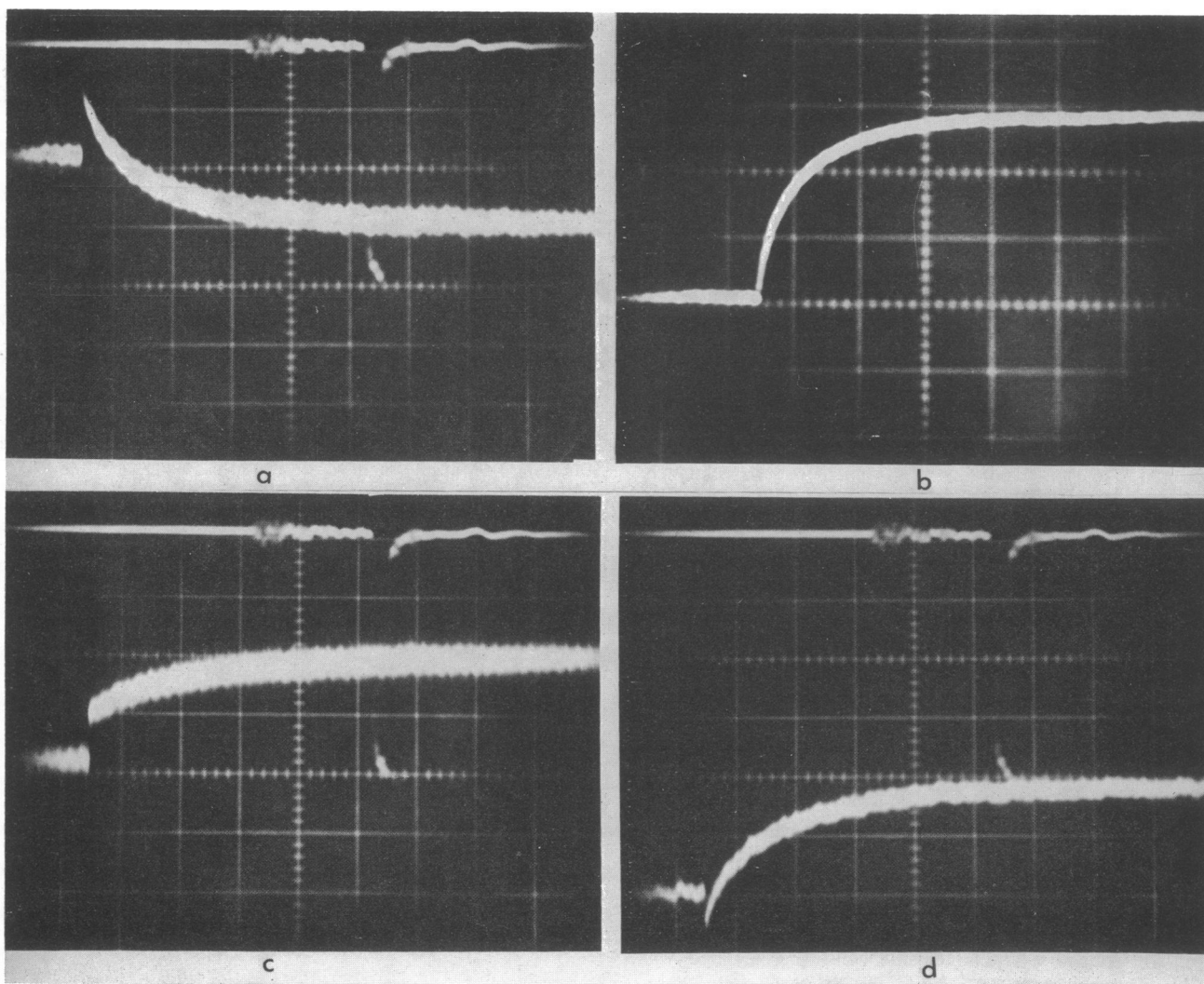
TABLE 1. *Specific rate constants of the reaction between hydrated electrons and cytochrome c*

Type of protein	$10^6 \times$ Fe(III)-cytochrome *c* M	$10^{-11} \times$ k_{700nm} M$^{-1} \cdot$sec^{-1}	$10^{-11} \times$ k_{370nm} M$^{-1} \cdot$sec^{-1}
Horse heart	0.5	1.7 ± 0.3	1.7 ± 0.3
Horse heart	1.0	1.3 ± 0.1	1.3 ± 0.1
Horse heart	4.0	1.2 ± 0.1	1.3 ± 0.1
P-551	1.0	0.9 ± 0.1	0.4 ± 0.05
P-551	5.0	1.0 ± 0.1	0.4 ± 0.05

Experiments at 20°, pH 6.0; no buffers or other salts were added.

FIG. 2. Absorption changes at pH = 6; [cytochrome c] = 1.0 × 10⁻⁵ M; dose = 200 rads. Band-width 2 nm. *Vertical* displacement corresponds to changes in transmission, *horizontal* to time. (a) = 405 nm, transmission, 3.3%/division; time, 100 msec/division. (b) = 425 nm, transmission, 5.2%/division; time 100 msec/division. (c) = 420 nm, transmission, 4%/division; time, 100 msec/division. (d) = 430 nm, transmission, 5%/division; time, 100 msec/division.

as illustrated in Fig. 2. In Fig. 2, the transmittance changes representing this slow transition are shown at different wavelengths. At 405 and 430 nm (a and d), an inversion in the direction of the transmittance change takes place, as expected from the spectra (Fig. 1). At 425 and 420 nm, only an absolute increase is observed, also in agreement with the two spectra.

The reduction yields of ferricytochrome c were determined at different dose rates and different initial ferricytochrome c concentrations. Maximal conversion of the produced e^-_{aq} was obtained at high ratios of ferricytochrome c to e^-_{aq} (10/0.7 μM). These yields were about 70% of the produced e^-_{aq}.

Alkaline solutions of horse heart cytochrome c

When alkaline (pH 11) solutions of ferricytochrome c were pulse radiolyzed, two separate phases of transmittance changes were recorded over the whole absorption range (Fig. 3). At a wavelength higher than 600 nm, the decay of e^-_{aq}

absorption is dominant. The specific rate of this decay is (1.0 ± 0.4) × 10¹⁰ M⁻¹ sec⁻¹ (i.e., significantly slower than the rate observed in neutral solutions). The faster phase of the transmittance changes below 600 nm was due to the reduction of ferricytochrome c, and had a kinetic pattern very similar to that of the e^-_{aq} decay. It also follows a pseudo first-order reaction, and the observed specific rate is (1.0 ± 0.3) × 10¹⁰ M⁻¹ sec⁻¹. The reduction yields of this fast phase are decreased to 12% of the produced e^-_{aq} (i.e., about 15% of the yield obtained under similar conditions in neutral solutions). The second slow phase of cytochrome c reduction takes place in the time range of hundreds of milliseconds, and its rate is independent of either protein concentration or total dose. The calculated specific first-order rate constant is 1.7 ± 0.3 sec⁻¹. The amplitude of this phase is significantly larger than that of the fast phase, and amounts to 20% of the produced hydrated electrons. Thus, the overall reduction yield in alkaline media is smaller than in neutral solution (about 30% of the e^-_{aq}).

Pseudomonas cytochrome *c* 551

In neutral solutions of P-551 cytochrome *c* transmittance changes were followed at 600 nm, were the e^-_{aq} band is dominant, and at 370, 400, and 550 nm, the cytochrome bands, the e^-_{aq} decay, and subsequent reduction of ferricytochrome *c*. Table 1 summarizes the calculated rate constants for the observed processes.

DISCUSSION

The reduction of horse-heart cytochrome *c* by hydrated electrons was shown (13) to proceed in a direct bimolecular process. The specific rates observed for the reactions were very close to the values calculated for the diffusion-controlled rate (13). Both absorption bands due to the hydrated electron and to the ferricytochrome *c* decay at the same synchronous rate. Though possible intermediate stages having life times shorter than 10^{-6} sec cannot be excluded by the equipment available to us, the data indicate that in the reduction by the e^-_{aq}, the electron is transferred directly to the exposed edge of the heme in the cytochrome *c* crevice.

The transient spectrum obtained 20 μsec after the pulse and the difference spectrum (reduced–oxidized) plotted according to the data of Margoliash and Frohwirt (19) are similar in shape, yet the transient is significantly blue-shifted. This spectral shift is most probably a result of the *ferro*-hemochrome being produced in the environment of a protein conformation that is still that of the ferric form. The pronounced differences between the conformation of ferri- and ferrocytochrome *c* have recently been resolved by x-ray crystallography at 2.45-Å resolution (3). Such conformational differences could easily account for alterations in the hemochrome environment. The coalescence of the transient and the static difference spectra is the result of the relaxation of the protein conformation to that of the equilibrium form of the ferrocytochrome *c*. The specific rate of this transition (8.5 sec^{-1}) is of the order of magnitude observed for many conformational transitions in protein molecules (20).

The change in the reactivity of ferricytochrome *c* in alkaline medium has been observed by Greenwood and Palmer (15). This effect was interpreted as being primarily due to a change in the ferri-heme site caused by a structural transition of the protein. A pH-dependent equilibrium between two conformations of ferricytochrome *c* has been inferred in several studies (15, 21); the conformation favored at high pH values was irreducible. The fast phase of the reaction of ferricytochrome *c* with e^-_{aq} represents the direct reduction of that fraction of the protein molecules present at equilibrium in the reducible conformation. The specific rate of the reaction in this phase is more than an order of magnitude smaller than that observed in neutral solutions. This effect should be due to the change in the net charge on the protein caused by the increase in pH. The electrostatic effect is also observed for the *Pseudomonas* cytochrome *c* 551, which is negatively charged in neutral solutions.

The second, rather slow phase of ferricytochrome *c* reduction is a monomolecular process. This is probably migration of the electron from its initial attachment site to the iron, and its rate-limiting step is the rate of transition of ferricytochrome *c* molecules into their reducible conformation. The dynamics of the conformational equilibrium of cytochrome *c* molecules in alkaline solutions were recently investigated by Schejter and Hess (ref. 22 and personal communication).
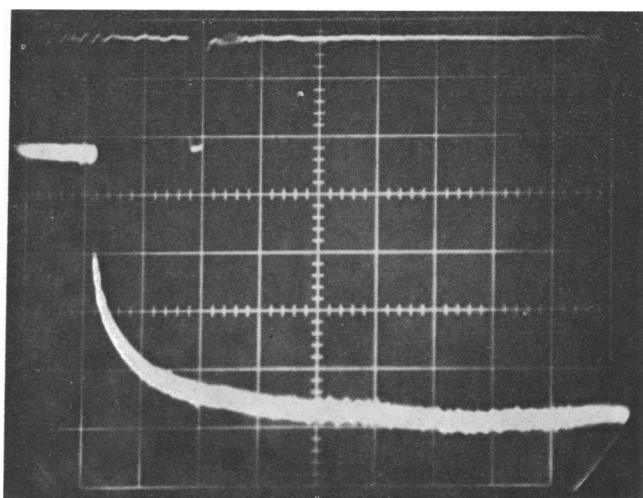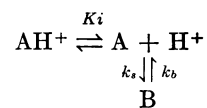

FIG. 3. Absorption changes at pH = 11, [cytochrome *c*] = 1.0×10^{-5} M; dose = 200 rads, λ = 360 nm. *Vertical* displacement corresponds to changes in transmission (1 large division = 1% transmission), *horizontal* displacement corresponds to time (1 large division = 500 msec).

They found that the system may be described by the following equation:

$$AH^+ \overset{Ki}{\rightleftharpoons} A + H^+$$
$$\underset{k_s \downarrow \uparrow k_b}{}$$
$$B$$

where AH^+, A, and B are the protonated reducible, unprotonated reducible, and irreducible conformations, respectively. Ki is the protonation equilibrium constant; k_s and k_b are the specific rates of the conformational transition. Thus, the observed rate of conformational change is:

$$k_{obs} = k_s \cdot Ki/([H^+] + Ki) + k_b.$$

When the values obtained by Schejter and Hess for Ki, k_s, and k_b (1×10^{-11} M^{-1}, 2.9 sec^{-1}, and 0.035 sec^{-1}, respectively) are introduced, the calculated value at pH 11 for the k_{obs} = 1.5 sec^{-1}. This value is in good agreement with the value obtained experimentally for the slow phase of the reduction (1.7 sec^{-1}) and confirms the assumption that it is the conformational transition that determines the rate of the second reduction phase.

The observed lower rate of reaction of P-551 cytochrome *c* with hydrated electrons is mainly a result of the net negative charge on these protein molecules. The observed specific rate of e^-_{aq} decay is again very close to that calculated from the Debye equation, when an average of a unit negative charge for the protein is assumed. Further experiments are required to explain the delay between the decay of the e^-_{aq} and the reduction of the ferriheme in this protein.

This study is a further illustration of the applicability of pulse radiolysis for study of the dynamics of biochemical electron-transfer processes. By use of the hydrated electron as reductant, the rather slow conformational changes following or controlling these reactions could be resolved.

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