THE REDUCTION OF CYTOCHROME *c* BY HYDRATED ELECTRONS

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1. Introduction

The technique of pulse radiolysis offers the possibility to produce hydrated electrons in aqueous solutions within the submicrosecond time range and to follow spectrophotometrically their reactions in the period following the pulse i.e. from 1 μsec to longer times [1–3]. The hydrated electron is a very potent reductant and constitutes a most elementary reagent in electron transfer reactions [3]. These advantages of the technique make it a rather effective tool for the study of electron transfer proteins. In a recent study of the reaction between the copper containing oxidase, laccase, and hydrated electrons, the electron accepting site in the enzyme was shown to be reduced by an indirect pathway at a rate much slower than the decay of the hydrated electrons [4]. This mechanism was assigned to the fact that the electron accepting Cu(II) in laccase is embedded in an hydrophobic surrounding, remote from any contact with the water molecules of the solvent [5]. In this report the rather different behaviour of cytochrome *c* is described. The hydrated electrons were found to reduce ferri-cytochrome *c* directly to the ferro state at a rate approaching the diffusion controlled limit.

2. Materials

Horse heart cytochrome *c*, obtained from Calbiochem, was purified on an Amberlite CG 50 column. All solutions were freshly prepared before each experiment in triply distilled water from which O_2 has been removed by continuous bubbling of purified argon. The pH was adjusted to 7.0 and no buffer was used. All other materials used were of analytical purity grade.

3. Methods

The experimental details involved in the pulse radiolytic technique have already been described [1, 2, 6]. All experiments were performed using the electron accelerator of the Hebrew University, Jerusalem. The reaction cell (4X2X1 cm) made out of fused quartz had a 12.5 cm optical path length [6]. Following the electrons pulse, the transmittance changes of the solutions were observed at three different wave lengths. This was done in order to track independently the changes in both oxidation states of the iron of cytochrome *c* (at 370 and 425 nm) and the decay of the hydrated electron (550 nm). Pulse lengths used were 0.1 μsec. Solutions were subjected to single pulse only and were changed subsequently.

* *Abbreviations:*
Cyt-c: Horse heart cytochrome *c*
e_{aq} : Hydrated electron

4. Results

Fig. 1 shows the oscilloscope traces of the transmittance changes due to: the decay of (a) the hydrated electrons (550 nm), (b) the ferri-cytochrome *c* (370 nm), the appearance of (c) the ferro-cytochrome *c* absorbancy at 425 nm. (The superimposed changes due to the parallel decay of the e_{aq} and ferri-cytochrome *c* may also be recognized).

Table 1 shows the observed bimolecular rate constants at different initial concentrations of ferri-cyt-c. The correlation between the rates observed at the different wave lengths clearly shows that the examined reaction is a direct reduction of the Fe(III) center of the protein by the hydrated electron. The rate constants of the reaction were also studied in the presence of different sodium chloride concentrations. The results of these measurements are presented in fig. 2. Cyt-c is known to be a basic protein molecule, i.e. positively charged over a wide pH range. The effect of increase in ionic strength is in accord with the expectation, namely, decreasing the specific rate constants. From the initial part of the slope it was possible to calculate the charge of the reacting protein molecule. The value was found to be close to +7; this value is in good agreement with other experimental values available in the literature [7].

Table 1
Rates of decay of the hydrated electrons and reduction of cytochrome *c*.

cyt-c ($M\times10^{-6}$)	$k_{550\ nm}$ ($M^{-1}\ sec^{-1}$)	$k_{425\ nm}$ ($M^{-1}\ sec^{-1}\times10^{-11}$)	$k_{370\ nm}$ ($M^{-1}\ sec^{-1}\times10^{-11}$)
0.5	1.9±0.4	1.9±0.5	1.9±0.4
1.0	1.3±0.1	1.4±0.3	1.4±0.2
2.0	1.3±0.1	1.3±0.3	1.3±0.1
3.0	1.2±0.1	1.3±0.3	1.2±0.1

All experiments carried out in the presence of 5×10^{-4} M NaCl and 5×10^{-2} M CH_3OH. t = 20±2°.

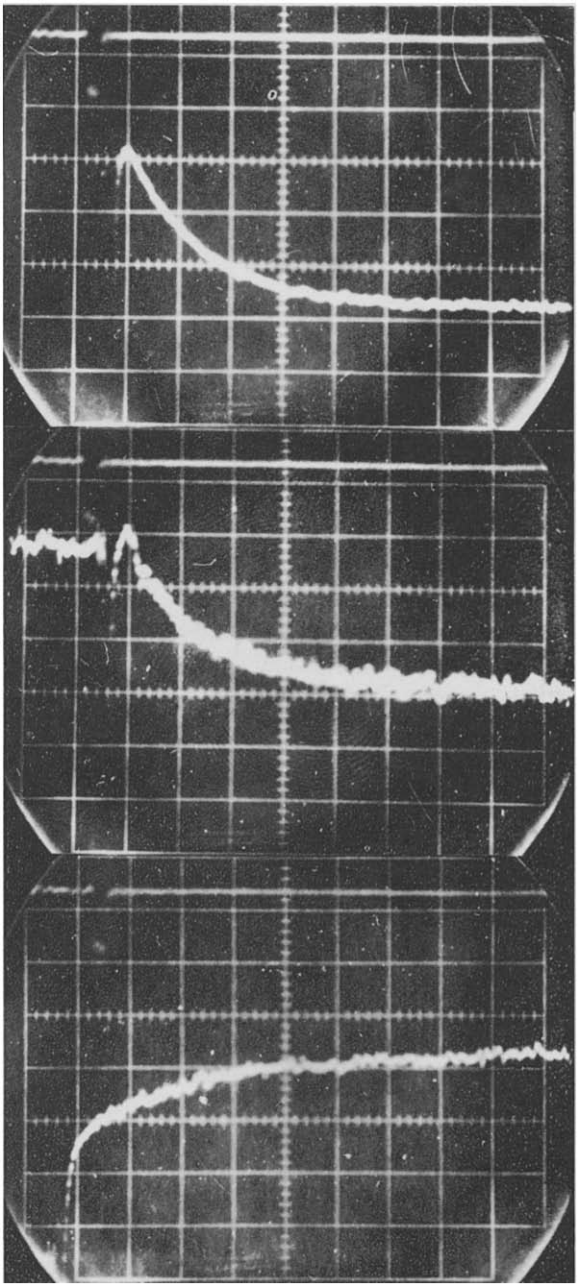

Fig. 1. Oscilloscope traces of the reduction of cyt-c by hydrated electrons. *Upper trace*: decay of e_{aq} followed at 550 nm. *Middle trace*: decay of ferri-cyt-c absorption observed at 370 nm. *Lower trace*: appearance of ferro-cyt-c absorption followed at 425 nm. For all three, sweep rates were 5 μsec/division.

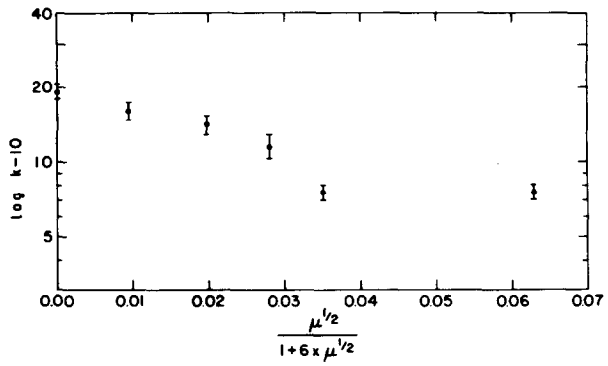

Fig. 2. The dependence of the logarithm of observed specific rate constants of e_{aq} with cyt-c on the square root of ionic strength.

5. Discussion

As our data clearly show, the reduction of ferricyt-c by hydrated electrons proceeds by a bimolecular diffusion controlled process. The synchronous decay of the e_{aq} and Fe(III)-cyt-c absorbancy and the appearance of the absorbancy due to the reduced form constitute the first example for specific and direct reduction of the electron mediation site of an electron transfer protein by e_{aq}. The specific rate constant of this reaction approaches the diffusion controlled limit calculated using Debye's equation [8]:

$$k_{dif} = 4\pi N(D_e + D_c)(r_e + r_c)Q/(e^Q - 1)$$

where N is Avogadro's number, D_e, D_c and r_e, r_c are the diffusion coefficients (47 and 1.3×10^{-6} cm^2 sec^{-1}) [2, 7] and radii (3 and 15×10^{-8} cm) [9] of the hydrated electron and cyt *c* respectively. $Q = -Z_c \times e^2/\epsilon kT(r_e + r_c)$ where Z_c is the charge of cyt-c, k is Bolzmann's constant and ϵ the dielectric constant of water. Introducing the appropriate values one obtains $k_{dif} = 3.4 \times 10^{11}$ M^{-1} sec^{-1}. From table 1 it is clearly seen that the observed rate constants do not fully reach the theoretically expected value. This difference might be an indication for the more refined steric requirements for the reduction of the Fe(III) ion. This indication is in fact substantiated by data from chemical modification studies and especially from the X-rays crystallographic structure analysis of this protein. It becomes evident that the heme group is surrounded by the hydrophobic side chains of the protein except for only one edge of it which is exposed by a crevice in the three dimensional structure [9], and two narrow channels leading from the surface to left and right sides of the heme.

The reaction pattern of copper oxidases, laccase and ceruloplasmin [4], with hydrated electrons is rather different from that observed for cyt-c. The electron mediating sites of the two former proteins seem to be embedded within these proteins and to be screened from the solvent water [5]. The hydrated electrons were therefore found to decay by reacting non specifically with the more accessible amino acid residues of the enzymes. The specific sites were reduced only in a second, inter-protein molecules electron transfer. This unique behaviour of the copper oxidases lends special weight to the assumption that an electron accepting site within a protein molecule could react rapidly with the hydrated electrons only when it is exposed to a sufficient degree. In the recent study by Braams and Ebert on the reaction of hydrated electrons with ribonuclease [10] the rate constants were found to increase as the macromolecule unfolds upon elevating the temperature. This behaviour was interpreted to be a result of the exposure of reactive groups being able to react with hydrated electrons.

The effect of ionic strength on the studied reaction is in line with that expected theoretically, at least over a limited range of salt concentrations. At higher ionic strength non linearity could be expected since the protein is known to bind anions with relatively great affinity [11]. Investigation of a possible correlation between this binding capacity and the reactivity are in progress.

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