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RADIOLYSIS OF CYTOCHROME *c*

EFFECT OF RADIOLYTICALLY GENERATED e_{aq}^- ON FERROCYTOCHROME *c*

K. J. H. VAN BUUREN, J. WILTING and R. BRAAMS

Physical Laboratory, Department of Molecular Biophysics, University of Utrecht, Sorbonnelaan 4, Utrecht (The Netherlands)

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INTRODUCTION

It is already known, that the main, initial product of the reaction of e_{aq}^- with excess ferricytochrome *c* is spectroscopically indistinguishable from native ferrocytochrome *c* and that it shows full biological activity [1–3].

Effects of larger doses of irradiation (i.e. where the total amount of reactant generated exceeds the amount of cytochrome *c* present) on the spectrum of cytochrome *c* have been studied by Stein and coworkers [4, 5]. However, quantitative data on the effects of the several reactants on the spectral and enzymic properties of the enzyme are lacking. We therefore started a further investigation on the effects of e_{aq}^- on the spectral and enzymic properties of cytochrome *c*.

MATERIALS AND METHODS

Horse-heart cytochrome *c* was isolated as described before [1, 2]. The concentration of the hemoprotein was determined spectrophotometrically, using a $\Delta A_{550\,nm}$ (red-ox) of 21 $mM^{-1} \cdot cm^{-1}$. $2 \cdot 10^{-5}$ M anaerobic neutral solutions of the ferric enzyme were irradiated in a ^{60}Co γ-source (Atomic Energy of Canada) at a rate of 61.5 rad/s. Aliquots taken at certain time intervals were examined for spectral and enzymic properties. Methanol in a 10^4 molar excess with respect to cytochrome *c* was present to scavenge the H · and OH · radicals, replacing them by methanol radicals.

RESULTS AND DISCUSSION

Fig. 1 shows the results of a particular experiment: the initial spectra do not differ from those of mixtures of native ferro- and ferricytochrome *c*: there is a growth of the α- and β-band, positioned at 550 and 520 nm, respectively, and isosbestic points at normal positions: 408, 505, 527, 540 and 557 nm. During this stage of the reaction

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Abbreviation: TMPD, *NNN'N'*-tetramethyl-*p*-phenylenediamine dihydrochloride.

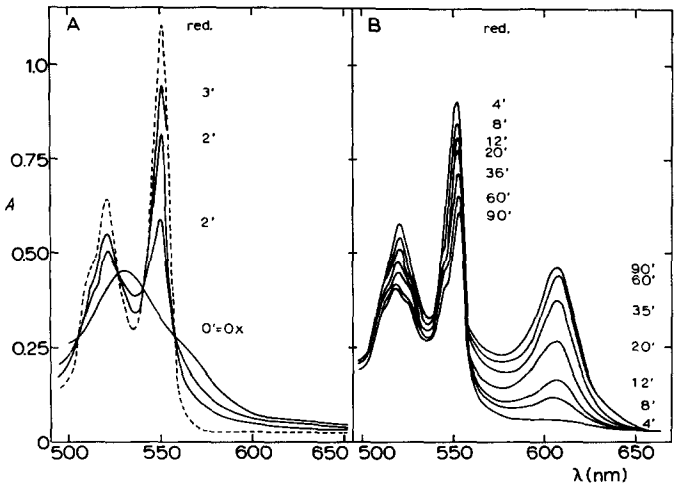

Fig. 1. Absolute spectra of cytochrome *c* obtained after irradiation of ferricytochrome *c* with ^{60}Co γ-radiation. The spectra were obtained with a Varian Techtron model 653 recording spectrophotometer at a scan speed of 10 nm/s. The cytochrome *c* stock solution (20 μM) was irradiated in an oxygen free 3.3 mM phosphate buffer (pH 7.2) containing 0.4 M methanol as a radical scavenger. Aliquots were taken at the times indicated in the figure and spectra were drawn immediately afterwards.

the enzymic activity in the ascorbate-TMPD-cytochrome *c*-cytochrome *c* oxidase-oxygen system is hardly affected by the radiation.

After a dose of 11 krad the 550-nm absorbance does no longer increase with increasing dose of radiation absorbed, although the value for 100 % reduction has not been reached. Moreover, the α-peak position has shifted somewhat towards higher wavelengths. When the irradiation is continued (Fig. 1B) the absorbance of the α-band decreases and its absorbance maximum shifts to 553 nm. The β-band absorbance decreases also but here the peak shifts towards lower wavelengths. Concomitant with the changes in absorbance observed at the α- and β-bands of ferrocytochrome *c* a broad banded absorbance, peaking around 606 nm, develops, while the enzymic activity decreases (cf, ref. 4). After 330 krad the reaction appears to be complete and a modified cytochrome *c* is obtained. This form of cytochrome *c* is characterized by absorbance maxima at 416, 517, 553 and 606 nm and has a biological activity of about 50 % of the unmodified enzyme. This irradiated cytochrome *c* is fairly stable towards auto-oxidation and does not show CO binding capacity.

To gain more detailed information about the reactions which cause the changes in spectral and enzymic properties we calculated the percentage of 550-nm absorbance (native enzyme = 100 %), the percentage of modification (taking $A_{606\ nm}$ after about 330 krad as 100 %) and the percentage remaining activity (native = 100 %) and plotted these data against the amount of e_{aq}^- produced per mole cytochrome *c* present. This is shown in Fig. 2. As can be seen, initially the degree of reduction of cytochrome *c* increases linearly with the amount of hydrated electrons generated. From the intersection of the extrapolated line with the 100 % line it can be calculated, that initially 1.04 hydrated electrons are needed for the reduction of 1 molecule of cytochrome *c*. The reduction yield for the reaction of hydrated electrons with ferri-

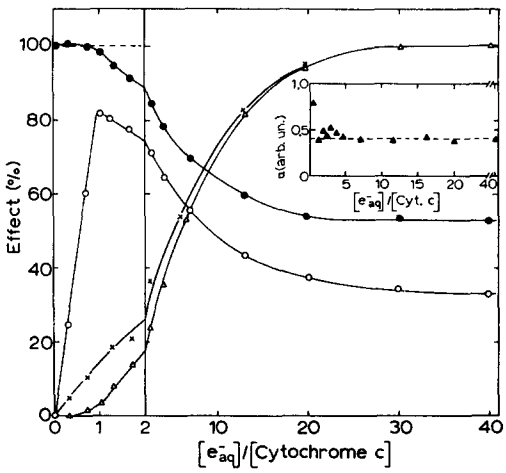

Fig. 2. Effect of the reaction of e_{aq}^- with ferricytochrome *c* on the spectral and enzymic properties of the enzyme. ○—○, 550-nm absorbance using oxidized enzyme; ●—●, 550-nm absorbance using reduced enzyme; △—△, 606-nm absorbance using oxidized enzyme and ×—×, 606-nm absorbance using reduced enzyme. For conditions see Fig. 1. Insert: Effect of the doses of radiation absorbed on α, the ratio $A_{606\,nm}/A$ activity.

cytochrome *c* in the ^{60}Co γ-source is thus slightly higher than the value of 0.85 reported for pulse-radiolysis experiments [2]. This apparent discrepancy could be ascribed to a slow reduction of cytochrome *c* by methanol radicals that in the steady-state radiolysis of the γ-source must be included but not in the pulse-radiolysis experiments [cf, ref. 6].

The production of modified cytochrome *c* shows a lag phase and begins when most of the cytochrome *c* has been reduced. Such a lag is also observed in the inactivation of the enzyme and it seems likely that the change in 606-nm absorbance and the decrease of the enzymic activity are related. We therefore calculated α, the ratio $A_{606\,nm}/A$ activity, and found that this ratio is independent of the time of irradiation (inset Fig. 2). It is therefore safe to assume that the occurrence of the 606-nm absorbance and the decrease of the enzymic activity are caused by the same modification reaction.

The presence of a lag phase might be interpreted in that reduced cytochrome *c* is involved in the modification reaction. This is supported by the observation that when ascorbate reduced cytochrome *c* is exposed to radiolysis the lag phase is absent. Then there are two possible explanations for the occurrence of the lag phase viz:

(1) the highly reactive ferric heme iron absorbs 80 % of the hydrated electrons produced (as deduced from the quantitative analysis of fast transients in pulse-radiolysis experiments) [2], so that initially relatively few electrons are available for modification reactions. After most of the heme is reduced, a larger proportion of the hydrated electrons becomes available for modification of ferrocytochrome *c*;

(2) the change in conformation caused by the change in redox state enhances the sensitivity of cytochrome *c* for modification by e_{aq}^-.

The first explanation appears the simpler one since it does not require further assumptions. However, our present results do not exclude the second possibility.

Most likely the occurrence of 606-nm absorbance is due to modification of the heme molecule itself rather than to a modification of the protein part, since the heme peptide obtained after proteolysis of the modified enzyme still shows this characteristic absorbance [7]. Absorbance peaks around 600 nm are observed in a-type hemes where a methyl side chain has been replaced by a formyl side chain [8], and in d-type hemes where one of the double bonds of a pyrrole ring is saturated [9]. A methanol radical (produced by the reaction of OH · or H · radicals with methanol) should be able to react with the heme. The particular situation with reduced cytochrome *c*, where the only heme edge available for attack by solvent molecules has a methyl and propionyl group already present, makes such a suggestion unlikely. The observation that replacement of methanol by t-butanol or formate as radical scavenger does not suppress the formation of modified cytochrome *c* (Van Buuren, unpublished observations) and the fact, that the γ-band position of cytochrome *c* is hardly shifted upon irradiation also points against the formation of formyl-containing heme.

The hydrated electron is a very powerful reducing agent ($E'_0 = -2.9$ V) [10]. It is therefore possible, that the spectral changes are caused by the reduction of a double bond of a pyrrole ring of the heme.

For electron transfer inside cytochrome *c*, it has been suggested that the electron leaves the heme iron via the heme π-system [11]. Saturation of a double bond could then decrease the effectivity of this electron transport and so explain the observed decrease in enzymic activity of the irradiated cytochrome *c* preparations. A further study of the radiation induced modification of cytochrome *c* may be useful in elucidating the electron carrier function of cytochrome *c*.

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