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A TUNNELLING MODEL TO EXPLAIN THE REDUCTION OF FERRICYTOCHROME *c* BY H AND OH RADICALS

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Summary

The kinetics of the reaction of OH radicals with ferricytochrome *c* was studied in the time range 1 μs to 1 s by means of pulse radiolysis. The OH radicals reduce ferricytochrome *c* by 40% ± 10%. The time course of the reduction is explained by a mechanism whereby a radical formed after hydrogen has been abstracted from the outer surface of the protein reduces the iron by electron tunnelling.

We have calculated that the reducing electron in the radical is bound with an energy of at least 1.75 eV and that the frequency factor of the tunnelling process is $\nu = 10^{11.5}\ s^{-1}$. This model accounts for the observed absorbance change in the time range $5 \cdot 10^{-6}$—10^{-1} s. The time course of the reduction of ferricytochrome *c* by H radicals (Lichtin, N.N., Shafferman A. and Stein, G. (1974) Biochim. Biophys. Acta 357, 386—398) is explained by the same model.

Introduction

When water is irradiated the following radicals are formed:

$H_2O \rightsquigarrow e^-_{aq}$ (2.8), OH· (2.8), H· (0.6).

The numbers between brackets denote the *G* values, i.e. the number of radicals formed per 100 eV absorbed.

When N_2O is present the hydrated electron is converted to OH radicals. The OH radical is a powerful oxidizing agent: the reduction potential of the couple $OH\cdot/H_2O$ at pH 7 is 1.8 V [1] or even higher [2].

Reducing radicals can be produced by hydrogen abstraction because many organic molecules (e.g. malate, lactate, ethanol) are able to reduce ferricytochrome *c* after they have reacted with H and OH radicals [3]. When an OH radical reacts with ferricytochrome *c* a similar reducing radical may be formed

on the surface of the molecule. To account for the high reactivity of H and OH radicals with cytochrome *c* ($k \geqslant 10^{10}\ M^{-1} \cdot s^{-1}$) it is assumed that many sites on the surface of the molecule will be available for these reactions [3]. Electrostatic repulsion or attraction can be neglected because H and OH radicals have no net charge.

Several authors [3—6], have investigated the reduction of ferricytochrome *c* by H and OH radicals. They concluded that intramolecular processes were involved, but they did not present theoretical calculations to explain the time course of the change in absorbance in their pulse radiolysis studies. In this paper we have derived a model to explain these processes which agrees rather well with the observed absorbance change. The basic assumption is that an electron coming from a reducing radical, produced by reaction with H and OH radicals and localized on the surface of a cytochrome *c* molecule, can tunnel through the protein towards the ferrihaem.

In order to explain the temperature dependence in tunnelling processes elaborate studies have been carried out by Grigorov and Chernavskii [7], Hopfield [8] and Jortner [9]. We used the same quasi-classical approximation for tunnelling as the authors of ref. 7, but we performed all of our experiments at constant temperature.

Materials and Methods

By means of pulse radiolysis coupled with fast kinetic spectroscopy we have measured the change in absorbance (A) in the time interval 10^{-6}—1 s after the pulse. Our solutions were irradiated with single 2-MeV electron pulses of 0.55 μs duration produced by a Van de Graaff accelerator. The cell forms part of a single beam spectrophotometer consisting of a 450 W Xenon lamp, two Bausch and Lomb grating monochromators (one in front of and one behind the cell), the irradiation cell (1.0 cm optical path length) and a 4840 RCA photomultiplier (response time < 100 ns). Changes in transmittance were recorded simultaneously by a transient digitizer (R 7912, Tektronix) and a Digital Processing Oscilloscope (DPO-system, Tektronix) with overlapping time bases.

Dosimetry was carried out with a Keithley electrometer which enabled us to measure the charge increment on the cell holder after each pulse. The increments were standardized against thiocyanate dosimetry (10 mM KCNS, oxygen saturated, $G \cdot \epsilon = 2.1 \cdot 10^4\ M^{-1} \cdot cm^{-1}$ at 480 nm [10]). The dose per pulse varied between 0.4 and 1.2 krad, corresponding to concentrations of 2.3—6.9 μM OH radicals per pulse in an N_2O-saturated solution (25 mM).

Argon (99.996%) was supplied by Hoek Loos, nitrous oxide (99.5%, chief impurity: nitrogen) by Air Liquide and phosphate was of analyzed reagent quality from J.T. Baker.

Deionized water was distilled four times in an all-borosilicate glass still to remove organic impurities. Horse ferricytochrome *c* was obtained from Sigma (type VI). It was used without further purification. Solutions containing 15—25 μM ferricytochrome *c* and 3 mM phosphate buffer (pH 7.0) were first deoxygenated by purging with argon for 1 h and were then saturated with nitrous oxide by purging for 45 min.

Using the rate constants $k(e^-_{aq}$ + ferricytochrome $c) = 4.5 \cdot 10^{10}\ M^{-1} \cdot s^{-1}$ and

$k(e^-_{aq} + N_2O) = 8 \cdot 10^9\ M^{-1} \cdot s^{-1}$ [11], it can be calculated that nearly all hydrated electrons (99%) react with N_2O, thereby producing OH radicals. Using $k(OH \cdot + \text{ferricytochrome } c) = 4 \cdot 10^{10}\ M^{-1} \cdot s^{-1}$ and taking into account the competition reactions $k(OH \cdot + OH \cdot) = 5 \cdot 10^9\ M^{-1} \cdot s^{-1}$ and $k(OH \cdot + H \cdot) = 1.2 \cdot 10^{10}\ M^{-1} \cdot s^{-1}$ [12] we calculated that more than 85% of the OH radicals had reacted with ferricytochrome *c*. The reaction was completed within 5 μs in all our experiments.

Since high doses were given in some of our experiments, some of the cytochrome *c* molecules reacted with two OH radicals. In these experiments no more than 20% of the cytochrome molecules that have reacted with OH radicals will react with two OH radicals. No marked difference was found between the absorption signals obtained at high dose and those obtained at lower dose.

Reduction of cytochrome *c* in our experiments is mainly caused by OH radicals, since the concentration of H radicals initially produced in our solution is about one order of magnitude lower than the concentration of OH radicals. Reduction of ferricytochrome *c* by OH radicals was observed at the wavelengths 550 and 425 nm. The bandwidth was 2 nm. All experiments were performed at room temperature (22 ± 1°C).

Theory

We shall consider the elementary model [7]. Two potential wells with radius r_1 and r_2 are separated by a potential barrier of width $b = a - r_1 - r_2$ and height V (see Fig. 1). E_{ki} and E_{kf} are the kinitic energies of the initial and final state. For convenience we shall consider that the barrier has a rectangular shape. The left well coresponds to the reducing radical formed on the surface of the cytochrome *c* molecule, the right well to the ferrihaem. The potentials within the wells will be considered constant. The barrier between the wells corresponds to the non-conducting protein layer. Initially the electron is in the left well and then passes into the right well (reduction of the ferricytochrome *c*). In general, there is an energy difference between the electron state in the left and in the right well; it is therefore necessary to compensate for this energy difference. This compensation may be effected through excitation or absorption of the vibrations of the surrounding medium. Elaborate calculations by the authors of ref. 7 gave an expression for the transition probability per unit time k:

$$k = \nu\, e^{-(2b/\hbar)\sqrt{2mV}} \tag{1}$$

where m is the mass of the electron. The frequency factor ν is a function of the temperature, of the change in quantum numbers of the vibrations, of the relative shifts in the equilibrium coordinates of the vibrations and of the level width of the excited vibratory states. More complex and detailed calculations concerning this frequency factor have been performed by Hopfield [8] and more recently by Jortner [9].

Because our experiments were performed at constant temperature we consider this frequency factor as a constant whose value has to be determined. Fig. 2 gives a schematic representation of the cytochrome *c* molecule. The molecule is taken as a sphere with radius r and the centre of the haem is located at distance d from the centre of the molecule. The angle θ varies between 0 and π.

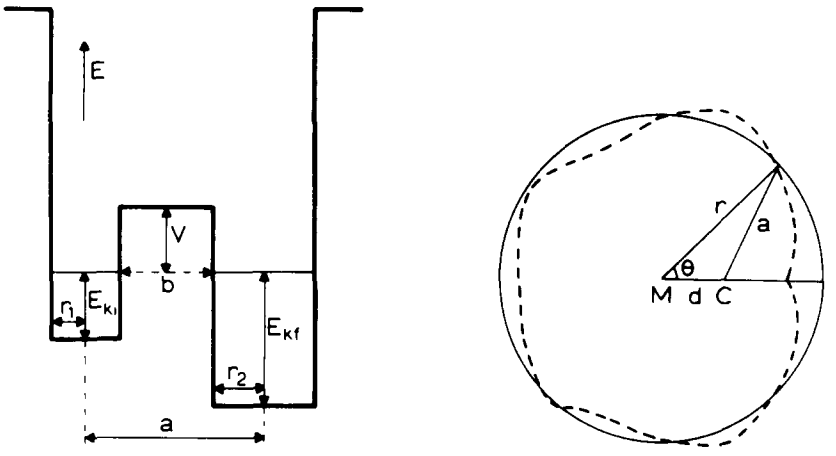


Fig. 1. A graphical representation of the two potential wells used to derive formula 2. The symbols are explained in the text.

Fig. 2. Graphical representation of the shape of the cytochrome *c* molecule (- - - - -) and the spherical approximation used to derive formula 3.

The distance between the two potential wells varies between $(r - d)$ and $(r + d)$ and there is rotational symmetry about the MC axis (see Fig. 2). By assuming that the reducing radical formed at distance a from the centre of the haem reacts with the haem according to first-order kinetics we obtained the following equation for the ratio of cytochrome *c* reduced after time t

$$c(t) = 1 - \int_{r-d}^{r+d} P(a)\, e^{-\nu t \exp(-\beta(a-r_1-r_2))}\, da \qquad (2)$$

where $\beta = \frac{2}{\hbar}\sqrt{2mV}$ and $P(a)da$ is the fraction of reducing radicals at a distance between a and $(a + da)$ from the centre of the haem.

If the radicals are distributed homogeneously at the surface of the cytochrome *c* the expression for $P(a)$ is:

$$P(a) = \frac{a}{2rd} \qquad (3)$$

The integral (Eqn. 2) can only be evaluated numerically; $c(t)$ was plotted versus $\log(\nu t)$ for different values of V (see Fig. 3). We used the following distances: for the radius r of the cytochrome *c* molecule the maximum value of 17 Å was taken, for the distance d between the centre of the haem and the centre of the molecule 5 Å [13], for the radius of the haem 5 Å and for the radius of the reducing radical 1 Å (see also Discussion).

Comparison of the theoretical curves with the experimental curve enables us to estimate V and ν. Calculations have shown that changes in r_1 and/or r_2 do not alter the shape of the theoretical curve (formulas 2 and 3) but affect only the frequency factor ν (see also Discussion).

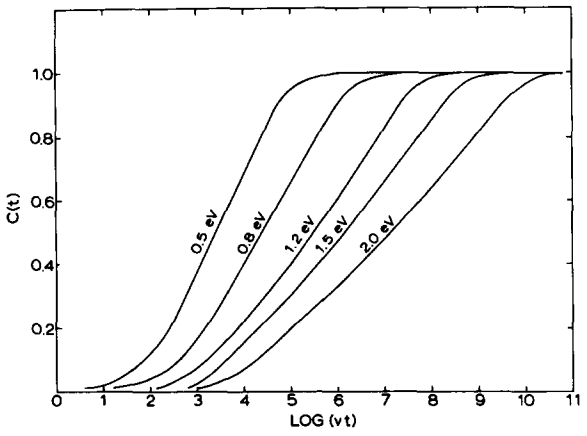


Fig. 3. Calculated curves according to formulas 2 and 3 for different values of the potential barrier.

Results

We found that the OH radicals had caused a 40% ± 10% reduction in ferricytochrome *c* at 550 nm as well as at 425 nm. This result was calculated by measuring the absorbance 100 ms after the pulse. With times longer than 100 ms the absorbance increased by about 10% (see Fig. 4).

We believe the change in absorbance after 100 ms is a result of the interaction of two cytochrome *c* molecules, one or both of which has or have been damaged by OH radicals.

The calculated curve (see Theory, formulas 2 and 3, and Fig. 3) could be fitted reasonably with the experimental data if the range of the measured absorbance were limited between 5 μs and 100 ms. Deviation from the theoretical curve during the first 5 μs is explained by the fact that the reaction of H and OH radicals with ferricytochrome *c* is still not complete.

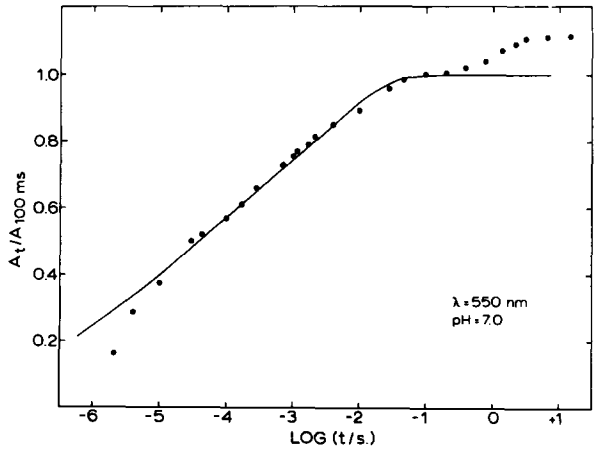


Fig. 4. Absorbance change at 550 nm (•) corresponding to the reduction of ferricytochrome *c* by OH radicals. The points are normalized to A_{100ms}. For experimental conditions see text. The solid line is calculated by using formulas 2 and 3 with $V = 1.75$ eV and $\nu = 10^{11.1}$ s^{-1}.

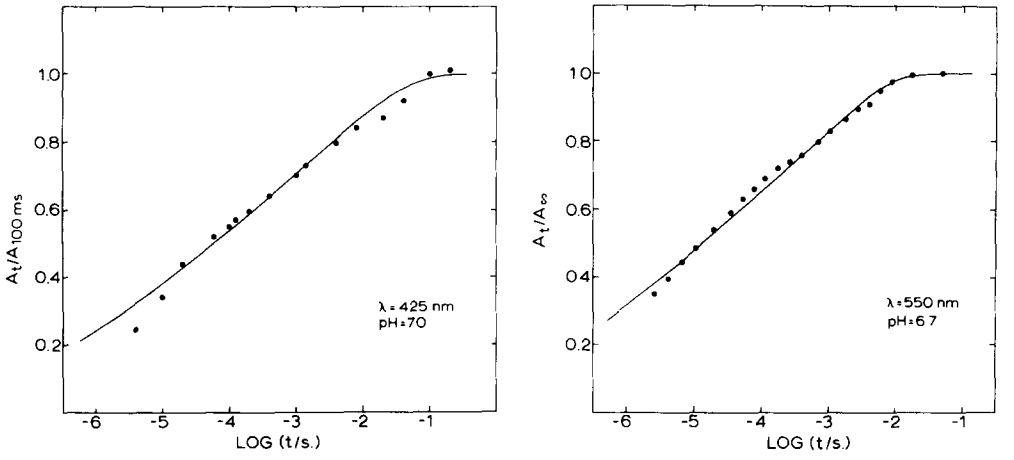


Fig. 5. Absorbance change at 425 nm (•) corresponding to the reduction of ferricytochrome *c* by OH radicals. The points are normalized to A_{100ms}. For experimental conditions see text. The solid line is calculated by using formulas 2 and 3 with $V = 2.0$ eV and $\nu = 10^{11.3}$ s^{-1}.

Fig. 6. Absorbance change at 550 nm (•) corrsponding to the reduction of ferricytochrome *c* by H radicals. The points are re-drawn after Fig. 2 of Lichtin et al. [5]. The ferricytochrome *c* and H radical concentration was 40 and 1.5 μM, respectively, and the dose 1.8 krad. The solid line is calculated by using formulas 2 and 3 with $V = 1.75$ and $\nu = 10^{11.5}$ s^{-1}.

Figs. 4 and 5 are both composed of at least four independent measurements. The solid lines in Figs. 4—6 are the absorbance changes calculated with formulas 2 and 3.

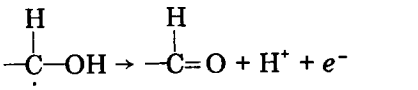
Fig. 6 shows the meaured points (redrawn after Fig. 2 of Lichtin et al. [5]), which represent the reduction of ferricytochrome *c* by H radicals, together with the best fit of our calculated curve (formulas 2 and 3). The best fit between the theoretical curve and our experimental data is obtained if $V = 1.75 \pm 0.25$ eV and $\nu = 10^{11.5\pm0.5}$ s^{-1}. At 425 nm the deviation from the theoretical curve is greater than at 550 nm. Consequently there is a greater uncertainty in the potential barrier ($V = 1.8 \pm 0.4$ eV). The frequency factor remains the same. For the reduction caused by H radicals (Fig. 6) the same values were obtained: $V = 1.75 \pm 0.25$ eV and $\nu = 10^{11.5\pm0.5}$ s^{-1}. Preliminary experimental studies of the reduction of human ferrihaemoglobin by OH radicals show a time dependence of the absorbance similar to that observed for ferricytochrome *c*. So we believe that for ferrihaemoglobin the same tunnel mechanism may apply.

Discussion and Conclusion

Reduction mechanism of radicals formed after hydrogen abstraction

For simple alcohols it is generally assumed that hydrogen abstraction by H and OH radicals takes place mainly at the α-carbon atom, thus forming a hydroxyalkyl radical [14,15]. In the absence of electron acceptors these radicals disappear because of dimerization and disproportionation. In the presence of e.g. Fe^{3+} the hydroxyalkyl radical is able to reduce the iron (ref. 14

and p. 138 of ref. 15). So the next reaction can be formulated:



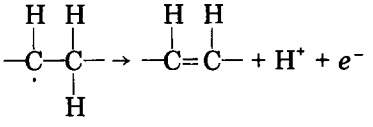
(4)

and the electron is transferred to the electron acceptor. In the case of disproportionation both electron and proton are transferred to another radical:

$$2R\dot{C}HOH \rightarrow RCHO + RCH_2OH \quad (5)$$

Reaction 4 explains the reduction of ferrihaemoproteins by hydroxyalkyl radicals as observed by e.g. Shafferman and Stein [3]. This reaction also explains why tertiary butanol does not reduce ferricytochrome *c* [3], for hydrogen abstraction occurs mainly on one of the methyl group [16] producing the radical $H_2\dot{C}C(Me)_2OH$. This radical probably only decays by dimerization because disproportionation (or reduction) would require fission of a C—C or C—OH bond, and such fission seems very unlikely [14].

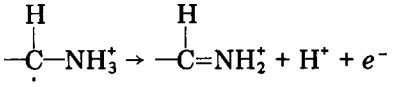
Because radiolysis of saturated hydrocarbons reveals that alkyl radicals disproportionate as well (e.g. p. 173 of ref. 15) we propose that it might be possible for alkyl radicals to reduce e.g. ferricytochrome *c* (see reaction 6)



(6)

OH radicals react with alkanes as well as with alcohols [12].

It is believed that in the presence of aliphatic amino acids OH radicals attack mainly the carbon atom that is alpha to the amino group. The α-amino radical may react by dismutation to form an imino-cation (Eqn. 7) which hydrolyzes to yield ammonia and a keto acid (Eqn. 8) (see ref. 12 and p. 235 of ref. 15).



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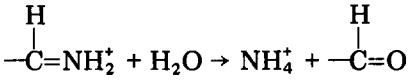

(8)

The cytochrome *c* molecule contains 19 lysines situated on the outside of the molecule. We believe that the lysines are the main source of production of reducing radicals formed after H abstraction.

Other groups on the outside of the molecule (e.g. aromatic groups, double bonds) react with H and OH radicals by a different mechanism, e.g. the radicals are added to the ring structure or double bond [12,15]. Because the reactivity of H and OH radicals with these groups is higher than, for example, for lysine a few of these groups (perhaps Tyr-74) on the outside of the molecule can cause a reduction of much less than 1.0. This should account for the observed reduction of about 40%.

By altering some of our parameters we were able to check how they influenced our results. It can be seen from formula 2 that changes in r_1 and r_2 affect

the frequency factor only. A decrease in r_2 (radius of the porphyrin) from 5 to 2 Å (distance Fe-N) increases the frequency factor by a factor 20.

A reduction in the radius of the molecule from 17 to 15 Å hardly changes the shape of the theoretical curve, but the frequency factor decreased by a factor 8.

The largest deviation between the real shape of the molecule and the spherical approximation occurs near the haem edge (shortest distance, see Fig. 2). This deviation affects the shape of the theoretical curve only during the first 5 μs after the pulse. Because the reaction of OH radicals with ferricytochrome *c* occurs in this time region as well, we have not taken into account any correction for the above-mentioned deviation.

The obtained value for the barrier height ($V = 1.75$ eV) is a lower limit because we have assumed a rectangular barrier. The height of the rectangular barrier can be seen as an average of the real potential shape. This minimum value is not unreasonable if we consider the energetics of the following reactions in which Fe^{3+}, $\dot{C}$-N represents ferricytochrome *c* directly after the reaction with OH radicals (carbon- and nitrogen-hydrogen bonds are omitted, see also reaction 7)

$$Fe^{3+}, \dot{C}—N \rightarrow Fe^{2+}, C{=}N + H^{+} \qquad (9)$$

$$\Delta G_0' = -25 — -50 \text{ kcal} \cdot M^{-1}$$

This $\Delta G_0'$ is calculated from the difference in reduction potential between the couples (C=N/$\dot{C}$—N) and (ferri/ferrocytochrome *c*). The value for E_0' (C=N/$\dot{C}$—N) is unknown but is estimated to lie between —1.6 V and —0.8 V [17]; E_0' (ferri/ferrocytochrome *c*) = 0.26 V [18]. The $\Delta G_0'$ value for reaction 10 is 71.5 kcal · M^{-1}, using E_0' (nH_2O/e_{aq}^-) = —2.9 V [15].

$$Fe^{2+}, C{=}N \rightarrow Fe^{3+}, C{=}N + e_{aq}^- \qquad (10)$$

For reaction 11 the value for $\Delta G_0'$ is +37.4 kcal · M^{-1} [19].

$$e_{aq}^- \rightarrow e^- + n\,H_2O \qquad (11)$$

Adding reactions 9, 10 and 11 we obtain

$$Fe^{3+}, \dot{C}—N \rightarrow Fe^{3+}, C{=}N + e^-$$

and

$$\Delta G_0' = +59—+84 \text{ kcal} \cdot M^{-1}.$$

This value corresponds to a maximum barrier height of 2.5—3.6 eV.

From our experiments we obtained $\nu = 10^{11.5}$ s^{-1}, which is rather low compared to the classically obtained frequency factor ($\nu = 10^{15}$ s^{-1}, which is the collison frequency of an electron against the wall of a potential well with depth $V \simeq 1$ eV). However, as Hopfield [8] suggested, the value of $\nu \simeq 10^{15}$ s^{-1} is certainly not correctly derived. Frank-Condon factors, vibronic coupling and Stokes shifts have to be taken into account. This makes our low value of ν quite reasonable.

Conclusion

In this paper we demonstrate that tunnelling may be an actual mode of intramolecular transmission of reducing equivalents, but alternative mechanisms cannot be ruled out. Another possibility is that the electron transfer may proceed by complex consecutive intramolecular reactions. Although the fit of the model to the data may be simply fortuitous, in our opinion the simple tunnelling model described in this article seems to give a fairly good explanation of the time dependence of the indirect reduction of ferricytochrome *c* by H and OH radicals.

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