Free Rad. Res. Comms., Vol. 6, No. 4, pp. 251–256
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THE MECHANISM OF CYTOCHROME *C* REDUCTION BY ALKYL RADICALS. EVIDENCE FOR MULTIPLE REACTION PATHWAYS

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(Received December 14, 1988)

The reactions of the hydroxyalkyl radicals $\cdot CH_2OH$ and $(CH_3)_2\cdot COH$ with oxidized cytochrome *c* are far more complex than previously reported. Analysis of the pulse-radiolytic data by kinetic modelling revealed that only about 40% of the alkyl radicals reduce the ferric iron chromophore. Altogether, four different reactions have to be considered for the disappearance of the alkyl radicals, only two of which affect the metal site. The data show that these radicals, similar to the much more reactive hydrated electrons and hydrogen atoms, are capable to react with biological macromolecules in diverse ways.

KEY WORDS: Methanol, isopropanol, hydroxyalkyl radicals, pulse radiolysis, rate constants, kinetic modelling.

ABBREVIATIONS: Cyt(II) – reduced cytochrome *c*, cyt(III) – oxidized cytochrome *c*.

INTRODUCTION

Oxidized cytochrome *c* (cyt(III)) is univalently reduced by a number of radicals. Rate constants for the most extensively investigated radicals hydrated electrons (e_{aq}^-, ref.[1,2]), hydrogen atoms (H·, ref.[2,3]) superoxide anions (O_2^-, ref.[4,5]) and formate radicals (CO_2^-, ref.[6]) are listed in compilations published by the Radiation Chemistry Data Center at Notre Dame University. Reactions with organic radicals were mostly limited to the methyl viologen (or paraquat) radical cation[7–9] and alcohol-derived alkyl radicals.[6]

For the radicals derived from methanol, ethanol and isopropanol, rather divergent rate constants were reported: $3 \times 10^7\,M^{-1}s^{-1}$ for $\cdot CH_2OH$,[10] $1.4–1.8 \times 10^8\,M^{-1}s^{-1}$ for $CH_3\cdot CHOH$,[10,11] and $2.6–3.8 \times 10^8\,M^{-1}s^{-1}$ for $(CH_3)_2\cdot COH$.[7,12,13] These radicals were proposed to react quantitatively with the ferric iron chromophore,[10,11] in contrast to e_{aq}^-[14] and H·[15] which reduce Fe(III) only to an extent of 30–50%. This behavior is to be expected in view of the structure of the protein, where only an edge of the porphyrin ring is exposed to the exterior.[16]

In the course of an investigation on the reactivity of hydroxyalkylperoxyl radicals with Cu, Zn-SOD we used cyt(III) as scavenger/competitor for O_2^-. As hydroxyalkyl radicals are the precursors of the respective peroxyl radicals (R. [1])[17]

$$\cdot R(OH) + O_2 \rightarrow R(OH)OO\cdot \qquad (1)$$

their reactions with cyt(III) under the condition of our experiments had to be re-investigated. Surprisingly, we found a very complex reaction pattern and far less than a stoichiometric reduction by $\cdot CH_2OH$ and $(CH_3)_2\cdot COH$ radicals.

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MATERIALS AND METHODS

Methanol and isopropanol (HPLC-grade, Baker) were used as 0.1 M solutions in N_2O-saturated 'Milli-Q' water (Millipore). N_2O was purged from residual O_2 by passing it through an Oxisorb column (Messer Griesheim). Cytochrome *c* (Boehringer Mannheim) was used as supplied.

The alkyl radicals were generated by 40 ns electron pulses from a Febetron 705 accelerator, details about the set-up were published elsewhere.[18] The kinetics of the absorption changes of cytochrome *c* were evaluated at 550 nm, using a $\Delta\varepsilon$ of $21\,000\,M^{-1}cm^{-1}$.[19] Kinetic modelling was performed analogous to previous descriptions.[20]

RESULTS AND DISCUSSION

Figure 1 shows a composite of four different experiments, reducing cyt(III) either with $O_2^{\cdot-}$ in the presence of the alcohols or with $\cdot CH_2OH$ and $(CH_3)_2\cdot COH$ radicals. Superimposed on the digitized experimental results are plots obtained by regression analysis or kinetic modelling over the whole time course of the reaction. It is evident

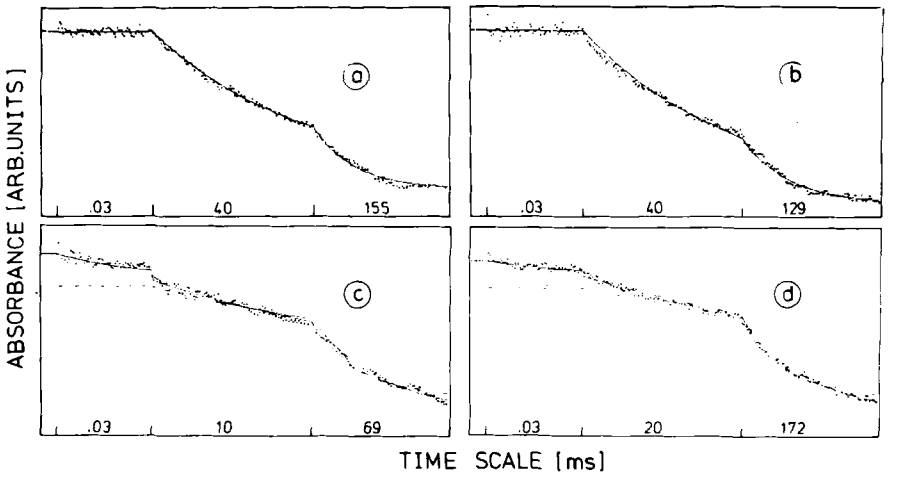

FIGURE 1 Kinetic plots for the reduction of cytochrome *c*(III) by $O_2^{\cdot-}$ (a, b) and by hydroxyalkyl radicals (c, d). Aqueous solutions at pH 8.0–8.5 (unbuffered), cytochrome *c*(III) concentration 20 μM. Data points are averages from three pulses; abscissae: ranges of observation periods at three different time resolutions in ms, ordinates: absorption changes in arbitrary units.
$O_2^{\cdot-}$-System: alcohol concentration 8 mM, sodium formate concentration 20 mM, O_2-saturated solutions; solid line is result of linearized regression analysis for pseudo-first order reaction.
(a) methanol: average pulse dose 7.9 Gy; signal strength 46 mAU; time resolution (T_1: 60 absorption values taken at 500 ns intervals for a total period of 0.03 ms in all plots), T_2: 100 × 0.4 ms = 40 ms, T_3: 86 × 1.8 ms = 154.8 ms; total observation time: 194.83 ms.
(b) isopropanol: 7.2 Gy; 43 mAU; T_2: 100 × 0.4 ms = 40 ms, T_3: 86 × 1.5 ms = 129 ms; total 169.03 ms.
$\cdot$R(OH)-System: alcohol concentration 100 mM, N_2O-saturated solutions; solid lines: plot of kinetic modelling results, dashed lines: results of regression analysis for pseudo-first order reaction.
(c) methanol: 7.7 Gy; 34 mAU; T_2: 100 × 0.1 ms = 10 ms, T_3: 86 × 0.8 ms = 68.8 ms; total 78.83 ms.
(d) isopropanol: 7.3 Gy; 40 mAU; T_2: 100 × 0.2 ms = 20 ms, T_3: 86 × 2.0 ms = 172 ms; total 192.03 ms.

that only $O_2^{\cdot-}$ attacks the ferric iron chromophore in a second-order reaction. The rate constant of $1.1 \times 10^6 M^{-1}s^{-1}$ in aqueous solution, pH 8.8, containing 8 mM of the alcohols is closest to the value of $1.4 \times 10^6 M^{-1}s^{-1}$ for the acidic form of cytochrome *c*.[21] Evidently the presence of the alcohol changes the dissociation behavior of the protein, as the form predominating above the pK (in aqueous solution) of 7.45 reacts far slower (21, cf. entry # 197 in ref.)[3] The fact, that no acceptable pseudo-first order fits are obtained from the reactions of the hydroxyalkyl radicals indicates that either a reaction sequence exists, governed by quite different rate constants or, alternatively, that several parallel and sequential reactions occur simultaneously.

To solve this problem we used a computer program for the simulation of fast chemical reaction kinetics[22] by an iterative approximation procedure. Using such an approach, we kept the mechanistic model simple enough, since otherwise mathematics might supersede chemistry and the results may become unreasonable!

As plausible mechanism for the attack of the hydroxyalkyl radicals we decided to take into consideration the intramolecular electron transfer processes, as they were suggested for e_{aq}^- [14] and H·:[15]

$$\cdot CH_2OH + Fe(III) \rightarrow Fe(II) + H^+ + NCHP \quad (2a)$$

$$(CH_3)_2\cdot COH + Fe(III) \rightarrow Fe(II) + H^+ + (CH_3)_2C{=}O \quad (2b)$$

$$\cdot CH_2OH + Protein \rightarrow \langle Protein\rangle^{\cdot-} + H^+ + HCHO \quad (3a)$$

$$(CH_3)_2\cdot COH + Protein \rightarrow \langle Protein\rangle^{\cdot} + H^+ + (CH_3)_2C{=}O \quad (3b)$$

$$\langle Protein\rangle^{\cdot-} + Fe(III) \rightarrow Fe(II) + Protein \quad (4)$$

Reactions (2a, b) represent the direct reduction of ferric iron by the alkyl radicals, whereas the combination of R. (3a, b) and R. (4) denote a reaction sequence in which an intermittently formed proteinyl radical anion (*vide infra*) transfers the electron to the iron chromophore in a first-order process. Introduction of these reactions led to an improvement of the fits, yet without fully satisfying the experimental data. Only the inclusion of a first-order scavenging reaction (R. (6)) in addition to the second-order decay (R. (5)):

$$\cdot R(OH) + \cdot R(OH) \rightarrow \text{non-radical products} \quad (5)$$

$$\cdot R(OH) \xrightarrow{(Protein)} \text{undefined scavenging} \quad (6)$$

to reduce the amount of alkyl radicals available for reduction of the chromophore, gave optimal fits with the experimental data points.

Table I lists the pertinent rate constants, obtained as average values for cyt(III) concentrations from 20–60 μM. Below that concentration cyt(III) was found insufficient for scavenging of the radicals (*vide infra*). All five rate constants were obtained as variables of the iterative optimization process, with fixed parameters being initial solvent concentrations, radical concentrations obtained from the respective pulse doses as well as the difference of the molar absorptivities between cyt(III) and cyt(II).

Using the rate constants of Table I, one may calculate the probability by which the alkyl radicals follow the individual reactions paths. As it turns out, the unspecific first-order decay (R. (6)) causes 42 or 44% of the alkyl radicals to disappear. With additional 17% to decay in the bimolecular process (R. (5)), only 39–41% of the radicals remain for reduction of the ferric iron. Of this remainder, about 20% follow reaction path (2) and 80% R. (3), making R. (3) the kinetically important observable

TABLE I
Rate constants contributing to the overall reduction of cytochrome *c* by alcohol-derived alkyl radicals

Alcohol	Rate constants				
	k_2 $\times 10^8\ M^{-1}\ s^{-1}$	k_3 $\times 10^8\ M^{-1}\ s^{-1}$	k_4 $\times 10^1\ s^{-1}$	2 k5 $\times 10^9\ M^{-1}\ s^{-1}$	k6 $\times 10^4\ s^{-1}$
Methanol	1.05 ± 0.15	4.59 ± 0.65	4.55 ± 0.4	1.60 ± 0.12	1.97 ± 0.05
Isopropanol	0.99 ± 0.15	3.92 ± 0.88	2.40 ± 0.7	1.68 ± 0.08	2.06 ± 0.35

Average values from 6–10 individual evaluations (each evaluation combined the data from three pulses) at pH 8.0–8.5 (without buffer) in 0.1 M alcoholic solutions.

reaction. Indeed, the rate constant (k_3) for $(CH_3)_2\cdot COH$ of $3.9 \times 10^8\ M^{-1}\ s^{-1}$ at pH 8.5 compares well to the previously determined value of $2.3 \times 10^8\ M^{-1}\ s^{-1}$ (above pH 8), respectively $3.6 \times 10^8\ M^{-1}\ s^{-1}$ below pH 7.[12] We consider the rate constant of $4.6 \times 10^8\ M^{-1}\ s^{-1}$ for $\cdot CH_2OH$ found in our study to be more reasonable than the unexpectedly low value of $3 \times 10^7\ M^{-1}\ s^{-1}$.[10]

Some of the reactions which we took into account merit further discussion. For instance, R. (3) as a *quasi* outer-sphere reaction could be written to denote a porphyryl radical anion to conform with similar proposals for iron-free porphyrin[23] and cytochrome P-450 reactions.[24] A protonated porphyryl radical anion has been reported to absorb at 408 nm,[23] close to the strong transient absorption at 418 nm, observed after e_{aq}^--attack.[25,26] However, owing to the very strong absorption of both cyt(III) and cyt(II) in this wavelength region in excess of $10^5\ M^{-1}\ cm^{-1}$,[16] no kinetic analysis could be performed as the concentration of the protein was too low for radical scavenging reactions.

Support for the involvement of protein rather than porphyryl radical anions in the sequence of R. (3) and (4) comes from the first-order electron transfer reaction to the ferric iron (R. (4)). It is a rather slow process with $45\ s^{-1}$ for methanol and $24\ s^{-1}$ for isopropanol and is the only reaction where the rate constants differ considerably for the two alcohols. It is thus more likely that they reflect conformation changes of the protein in the 0.1 M alcoholic solutions – the overall reactivities of the two radicals being quite similar. Indeed, the values are reasonably close to the reported first-order rate constant of $30\ s^{-1}$ for the opening of the heme pocket[27] after reduction by dithionite – a reaction which probably involves the SO_2^- radical.[28,29] In contrast, the intramolecular first-order process after e_{aq}^--attack occurs with a rate constant of $1.2–1.3 \times 10^5\ s^{-1}$.[14,26] According to our kinetic model, this conformation change is evidently induced by the radical attack at the protein, at a site close to the heme crevice.

Reaction (5) represents the bimolecular decay of the $\cdot R(OH)$ radicals, the closely similar values of 1.6 and $1.7 \times 10^9\ M^{-1}\ s^{-1}$ are in agreement with the reported values of 2.4 and $1.4 \times 10^9\ M^{-1}\ s^{-1}$, respectively.[30] The final reaction (R. (6)) which we had to incorporate for optimization of our fits with the experimental data, is represented in the kinetic model by a first-order process. Only if we assume this reaction to reflect scavenging of the radicals at localized sites of the protein without affecting the chromophore group (thus being transparent to optical analysis), should it be dependent on the concentration of the protein. As this was not observed, we propose R. (6) to represent a unspecific destruction of the hydroxyalkyl radicals on the surface of the protein, in which case a dependence would only be apparent over a much wider concentration range. This reaction is in complete contradiction to the reported fully

stoichiometric reaction of methanol[10] and ethanol alkyl radicals,[10,11] but its omission from the kinetic model grossly distorted the plots. It is interesting to note that the reaction of $\cdot CH_2OH$ with a Fe(III)-poryphyrin complex[31] also conforms to a 1:1 stoichiometry. In this case, however, the rate constants of $9 \times 10^8 M^{-1}s^{-1}$ at pH 8.1 and $1.4 \times 10^9 M^{-1}s^{-1}$ at pH 5.6 do suggest diffusion-controlled attack at the chromophore.

We have, in our kinetic model, neglected that attack of ·OH radicals forms only 95.5% $(CH_3)_2\cdot COH$ α-radicals (13.3% are $\cdot CH_2(CH_3)CHOH$ or β-radicals and the rest of 1.2% alkoxyl radicals from attack at the hydroxyl group[32]). The reducing capacity of the two alkyl radicals may be somewhat different, but looking at the generally similar rate constants of $\cdot CH_2OH$ and $(CH_3)_2\cdot COH$ in our system, the difference probably would be too small to have an effect. A recent study on the reaction of SO_4^- with isopropanol,[33] based on analogous kinetic modelling, suggests that the β-radical is slowly converted into the α-radical in a bimolecular reaction with the parent alcohol.

In conclusion, the reactions of the hydroxyalkyl radicals $\cdot CH_2OH$ and $(CH_3)_2\cdot COH$ with oxidized cytochrome *c* are more complex than previously reported. Four separate decay reactions can be distinguished, only two of which affect the ferric iron chromophore. Altogether, only about 40% of the alkyl radicals formed by the electron pulse are effectively reducing the ferric iron. The fact that previously determined rate constants agree with the corresponding values from our kinetic model shows the reliability of such an approach. It also demonstrates that alkyl radicals, similar to the far more reactive primary radicals from water, e_{aq}^-, ·OH and H·, are equally capable of reacting with proteins in manifold reaction pathways. It is thus likely that radical inactivation of other biological macromolecules also conforms to such a pattern.

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Accepted by Prof. H. Sies