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REDUCTION OF FERRICYTOCHROME *c*, METHEMOGLOBIN AND METMYOGLOBIN BY HYDROXYL AND ALCOHOL RADICALS

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Summary

We have studied the reaction of ferricytochrome *c*, methemoglobin and metmyoglobin with OH and alcohol radicals (methanol, ethanol, ethylene glycol and glycerol). These radicals can be divided into three groups:

1. The OH radicals which reduce the ferricytochrome *c* with a yield of (30 ± 10)% and methemoglobin with a yield of (40 ± 10)%. They do not reduce metmyoglobin. The reduction is not a normal bimolecular reaction but is most probably an intramolecular electron transfer of a protein radical.

2. Methanol and ethanol radicals which reduce all three hemoproteins with a yield of (100 ± 5)%. This reduction is a normal bimolecular reaction.

3. Glycerol radicals which do not reduce the ferrihemoproteins under our experimental conditions. Ethylene glycol radicals do not reduce ferricytochrome *c* and metmyoglobin but they do reduce methemoglobin with a yield of (30 ± 10)%.

Introduction

The reduction mechanism of ferricytochrome *c* has attracted much attention [1—6] because of the biochemical function of cytochrome *c* in the mitochondrial respiratory chain. Recently the reduction mechanism of other ferrihemoproteins has also become a subject of interest [7,8]. We have therefore compared the reduction of ferricytochrome *c* with the reduction of methemoglobin and metmyoglobin.

Myoglobin, cytochrome *c* and the monomer of hemoglobin are similar in size and shape and they all have a heme group which is in contact with the solvent.

We have studied the reaction of these hemoproteins with OH radicals and radicals formed after hydrogen abstraction from methanol, ethanol, ethylene glycol and glycerol. The radicals were produced by pluse- and γ-radiolysis in dilute aqueous solutions. The G-values for the products of radiolysis of water are: $G(e^-_{aq}) = 2.8$, $G(OH) = 2.8$, $G(H) = 0.6$, and $G(H_2O_2) = 0.7$. At high alcohol concentrations (≈ 1 M), the alcohol scavenges OH radicals from the spurs as well. In an N_2O saturated solution we have used as value for the production of alcohol radicals of 6.5 (hydrogen atom included).

Materials and Methods

Horse heart ferricytochrome *c* (type VI) and sperm whale metmyoglobin (type II) were obtained from Sigma and were used without further purification. Human ferrihemoglobin was prepared as described [9]. The various hemoproteins were diluted in a 3.3 mM phosphate buffer (pH 7). Water was triply distilled. The concentration varied between 30—60 μM (on the heme basis) for methemoglobin and between 10—20 μM for ferricytochrome *c* and metmyoglobin. The concentration of the alcohols varied between 0.1 and 1.0 M. The alcohol concentration was always sufficient to scavenge practically all the OH and H radicals. All chemicals used were of analyzed reagent quality and were obtained mainly from J.T. Baker (Deventer, The Netherlands).

The solutions were deaerated by bubbling with high purity argon for 45 min. and then saturated with N_2O for 30 min. In the presence of N_2O the hydrated electrons are converted into OH radicals.

In order to determine the yield of reduction the solutions were irradiated with different doses in a ^{60}Co γ-source (Gammacell 200, Atomic Energy, Canada; dose rate 1.7 krad/min.). The dose was varied between 0.1—25 krad. After irradiation the methemoglobin and metmyoglobin solutions were saturated with oxygen and the difference spectrum between the solution before and after irradiation was recorded. When this difference spectrum agreed with the difference spectrum between the met- and oxyform, the absorbance difference at 542 nm was put in a graph against dose. For cytochrome *c* the absorbance difference at 550 nm was used. In the presence of methanol and ethanol we obtained a straight line while for the reduction by OH radicals the line was slightly curved (less reduction with increasing dose). From the slope extrapolated to zero dose we obtained the reduction efficiency. We used for hemoglobin (on the heme basis) and myoglobin ΔA_{542nm} (oxy-met form) = 7 $mM^{-1} \cdot cm^{-1}$ and for cytochrome *c* ΔA_{550nm} (red-ox) = 21 $mM^{-1} \cdot cm^{-1}$.

To determine the rate constant for the reaction of the hemoproteins with methanol- and ethanol-radicals we used the pulse-radiolysis technique. The electron pulse had a duration of 0.5 μs and was produced by a 2 MV Van de Graaff accelerator (High Voltage Engineering, Europe). The optical detection system has been described previously [9].

Dosimetry was carried out with a 10 mM KCNS solution, oxygen saturated with $G \cdot \epsilon = 2.1 \cdot 10^4$ $M^{-1} \cdot cm^{-1}$ at 480 nm. The pulse dose varied between 1—6 krad. All experiments were performed at room temperature (22 ± 1°C).

Results and Discussion

The results are summarized in Tables I and II. The values listed are the average of at least three independent measurements.

Reduction by OH radicals

The reduction by OH radicals of cytochrome *c* has been investigated by many authors [4,6,10,11]. They found a reduction yield varying between 25 and 55%. We have recently proposed an electron tunnel mechanism to explain the observed kinetics [11]. For hemoglobin we have observed similar kinetics and reduction yield, so we believe the reduction process to be the same for both ferricytochrome *c* and methemoglobin. For methemoglobin we also found a large spread in the reduction yield. Several factors may be responsible:

(a) The radical formed on the protein part after the reaction of an OH radical may react with solute molecules. Therefore the yield may be dependent on the kind of buffer, its concentration and the concentration of the hemoproteins. By interaction with two hemoprotein molecules one molecule can be reduced by a radical formed on the other.

(b) Upon continuous irradiation the hemoproteins will be damaged.

(c) Hydrogen peroxide formed by radiolysis can oxidize the reduced hemoproteins. Therefore the reduction efficiency is dependent on the irradiation time (dose rate) and the time between irradiation and the recording of the spectrum.

For cytochrome *c* we have neglected the influence of H_2O_2 because no deviation in the spectra were found. For methemoglobin γ-irradiated with more than 4 to 5 krad we found a deviation from the oxy spectrum (e.g. absorbance increase at 630—650 nm). This means other spectra besides the met- and oxy spectrum are involved. The other spectra may be due to molecules which are damaged and cannot bind oxygen any more, but hydrogen peroxide may also be responsible. Formation of a ferryl complex by H_2O_2 [12,13] cannot explain the total deviation so hemichrome formation after reaction of H_2O_2 with deoxy hemoglobin may also contribute [14].

The rate constant for the reaction of OH radicals with ferricytochrome *c* and methemoglobin has already been published [15]. For metmyoglobin we have assumed that the rate constant with OH radicals is the same as for ferricytochrome *c*. After the reaction of OH radicals with metmyoglobin and after

TABLE I

THE REDUCTION EFFICIENCY OF THE RADICALS WITH THE FERRIHEMOPROTEINS

Conditions: 3.3 mM phosphate buffer (pH 7), oxygen free, N_2O saturated.

Radical	Cytochrome *c*	Hemoglobin	Myoglobin
OH	0.30 ± 0.10	0.40 ± 0.10	<0.05
Methanol	1.00 ± 0.05	1.00 ± 0.05	0.95 ± 0.05
Ethanol	1.00 ± 0.05	1.00 ± 0.05	0.95 ± 0.05
Ethylene glycol	0.0 ± 0.03	0.30 ± 0.10	<0.05
Glycerol	0.0 ± 0.03	0.0 ± 0.03	<0.05

TABLE II

THE RATE CONSTANT ($M^{-1} \cdot s^{-1}$) FOR THE REACTION OF THE RADICALS WITH THE FERRIHEMOPROTEINS

Conditions: 3.3 mM phosphate buffer (pH 7), oxygen free, N_2O saturated. The rate constant of methanol and ethanol radicals with ferrihemoglobin is based on the heme concentration.

Radical	Cytochrome *c*	Hemoglobin	Myoglobin
OH	$1.4 \cdot 10^{10}$	$3.6 \cdot 10^{10}$	$\approx 1.5 \cdot 10^{10}$
Methanol	$(3.0 \pm 0.5) \cdot 10^7$	$(9.5 \pm 1.5) \cdot 10^6$	$(2.4 \pm 0.5) \cdot 10^7$
Ethanol	$(1.4 \pm 0.2) \cdot 10^8$	$(4.0 \pm 0.4) \cdot 10^7$	$(5.5 \pm 0.5) \cdot 10^7$

saturating the solution with oxygen we do not find any sign of oxymyoglobin. The observed difference spectrum, which increases with increasing dose, can also be obtained by adding small amounts of hydrogen peroxide, and is ascribed to the formation of a ferryl complex [12,13]. We have therefore concluded that OH radicals do not reduce metmyoglobin.

In general after hydrogen abstraction a reducing radical is formed. This radical is able to reduce the iron by intramolecular processes or tunneling [11]. Addition to double bonds and aromatic groups will normally not give a reducing radical. This may explain why the reduction efficiency for OH radicals is less than 1.0 for cytochrome *c* and hemoglobin. One reason why OH radicals do not give any reduction of metmyoglobin may be the latter's low reduction potential (0.046 V for myoglobin, 0.17 V for hemoglobin and 0.25 V for cytochrome *c*) [16].

Upon irradiation H radicals are formed as well. However they reduce cytochrome *c* with the same kinetics and with approximately the same yield as OH radicals [2,3]. For this reason and because in a N_2O saturated solution $G(H) \approx 0.1\ G(OH)$ we have not distinguished between H and OH radicals.

Reduction by methanol- and ethanol radicals

Both radicals reduce the hemoproteins by approximately 100%. This reduction can be explained by the following reaction mechanism [17].

$$\mathrm{R{-}\overset{\displaystyle\cdot}{\underset{\displaystyle H}{C}}{-}OH + Fe^{3+} \xrightarrow{k_1} R{-}\underset{\displaystyle H}{C}{=}O + Fe^{2+} + H^+} \qquad (1)$$

where Fe^{3+} denotes a ferrihemoprotein.

For this reaction it is necessary that the radical interacts directly with the heme group. The reduction potential for methanol and ethanol radicals is $E_0(H^+ + CH_2O/CH_2OH) = -0.97$ V and $E_0(H^+ + CH_3CHO/CH_3CHOH) = -1.1$ V [18]. This can partly explain the higher rate constant for ethanol radicals (see Table II). To measure the rate constants we have used the pulse-radiolysis technique. After the pulse the following equations hold

$$dA/dt = -k_1 A \cdot R \qquad (2)$$

$$dR/dt = -k_1 A \cdot R - 2k_2 R^2 \qquad (3)$$

where A denotes the ferrihemoproteins concentration and R the alcohol radical concentration.

Preliminary experiments have shown that $k_1 \leqslant 0.1\ (2k_2)$. If $[A] = 15\ \mu M$ and $[R]_{t=0}$ is 20 to 40 μM then $k_1 A << 2k_2 R$ during the first 80% of the decay. Now Eqn. 3 can be solved. Substitution of the solution in Eqn. 2 gives

$$\ln(A(t)/A_0) = -(k_1/2k_2) \cdot \ln(1 + 2k_2 R_0 t)\ . \qquad (4)$$

For methanol and ethanol $2k_2 = 2.3 \cdot 10^9\ M^{-1} \cdot s^{-1}$ [19]. By using the first 80% of the observed absorbance change at 550 and 435 nm we obtained a straigth line in a double logarithmic plot (see Eqn. 4) from which k_1 was calculated (Table II).

The rate constant of ethanol radicals with ferricytochrome *c* obtained in this way agreed very well with the value given by Shafferman and Stein [2] ($k_1 = [1.8 \pm 0.2] \cdot 10^8\ M^{-1} \cdot s^{-1}$).

Irradiation in the presence of ethylene glycol and glycerol

In agreement with several authors [5,20] we have found that the ethylene glycol and glycerol radicals do not reduce ferricytochrome *c*. These radicals also do not reduce metmyoglobin. After γ-irradiation of metmyoglobin there is however a build up of a characteristic low spin spectrum. This spectrum can also be obtained by adding small amounts of hydrogen peroxide and is due to the formation of a ferryl complex [12,13]. The build up of the spectrum agrees with the total amount of hydrogen peroxide formed during γ radiolysis. Ethylene glycol radicals reduce methemoglobin with a yield of $(30 \pm 10)\%$ but glycerol radicals do not reduce methemoglobin.

It is known that ethylene glycol radicals can decay by a dehydration reaction [21]

$$CH_2OH\dot{C}HOH \xrightarrow{k_5} \dot{C}H_2CHO + H_2O \qquad (5)$$

Polarographic studies have shown that the radical formed after dehydration is a much weaker reducing species than its precursor [21].

By pulse radiolysis we have studied the decay of ethylene glycol and glycerol radicals in an N_2O saturated (oxygen free) solution around pH 7. In the presence of glycerol (1 M) we have observed at 280 nm a first order decay to a species which is stable for at least 100 ms after the pulse. This first order process ($k = 5 \cdot 10^3\ s^{-1}$) we ascribe to the dehydration of the glycerol radical. However, in the presence of ethylene glycol we found a normal second order decay of the radical due to dimerisation ($2k = 7 \cdot 10^8\ M^{-1} \cdot s^{-1}$, pulse dose 3—6 krad). We therefore concluded that the dehydration reaction for ethylene glycol radicals at pH 7 must be less or equal to $10^3\ s^{-1}$.

The dehydration of the polyalcohol radicals does not mean that these radicals are unable to reduce ferrihemoproteins. For glycerol radicals the dehydration reaction will dominate the reduction of the ferrihemoproteins if the rate constant for reduction does not exceed $10^7\ M^{-1} \cdot s^{-1}$. We therefore believe that the high rate constant of dehydration of the glycerol radicals can explain why these radicals do not reduce ferrihemoproteins.

We do not know whether this argument also holds for ethylene glycol radicals

which have a lower dehydration rate than glycerol radicals. The question why ethylene glycol radicals reduce methemoglobin with a yield of 30% but do not reduce metmyoglobin and ferricytochrome *c* can not be answered at this moment. More experiments will be necessary to elucidate this problem.

The presence of a high concentration of alcohols will have some effect on the hemoproteins. In the presence of alcohols the pK_a value of cytochrome *c* is lowered [22]. For hemoglobin the dissociation equilibrium of tetramers into dimers will be changed and in general the hemoprotein structure will be more open. We believe that under our experimental conditions these changes in the hemoproteins will have no drastic effects on the reduction yields and rate constants determined in this article.

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