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# THE KINETICS OF THE REDUCTION OF CYTOCHROME *c* BY THE SUPEROXIDE ANION RADICAL

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## SUMMARY

1. At neutral pH ferricytochrome *c* is reduced by the superoxide anion radical ($O_2^-$), without loss of enzymatic activity, by a second order process in which no intermediates are observed. The yield of ferrocytochrome *c* (82–104 %), as related to the amount of $O_2^-$ produced, is slightly dependent on the concentration of sodium formate in the matrix solution.

2. The reaction ($k_1 = (1.1 \pm 0.1) \cdot 10^6\ M^{-1} \cdot s^{-1}$ at pH 7.2, $I = 4$ mM and 21 °C) can be inhibited by superoxide dismutase and trace amounts of copper ions. The inhibition by copper ions is removed by EDTA without interference in the $O_2^-$ reduction reaction.

3. The second-order rate constant for the reaction of $O_2^-$ with ferricytochrome *c* depends on the pH of the matrix solution, decreasing rapidly at pH > 8. The dependence of the rate constant on the pH can be explained by assuming that only the neutral form of ferricytochrome *c* reacts with $O_2^-$ and that the alkaline form of the hemoprotein is unreactive. From studies at pH 8.9, the rate for the transition from the alkaline to the neutral form of ferricytochrome *c* can be estimated to be 0.3 $s^{-1}$ (at 21 °C and $I = 4$ mM).

4. The second-order rate constant for the reaction of $O_2^-$ with ferricytochrome *c* is also dependent on the ionic strength of the medium. From a plot of log $k_1$ versus $I^{\frac{1}{2}} \cdot (1+\alpha I^{\frac{1}{2}})^{-1}$ we determined the effective charge on the ferricytochrome *c* molecule as +6.3 and the rate constant at $I = 0$ as $(3.1 \pm 0.1) \cdot 10^6\ M^{-1} \cdot s^{-1}$ (pH 7.1, 21 °C).

5. The possibility that singlet oxygen is formed as a product of the reaction of $O_2^-$ with ferricytochrome *c* can be ruled out on thermodynamic grounds.

## INTRODUCTION

Upon irradiation of water with high energy photons or electrons the following radicals and molecules are formed:

$$H_2O \rightarrow e_{aq}^-,\ OH^{\cdot},\ H^{\cdot},\ H_2,\ H_2O_2,\ H^+ \text{ and } OH^- \qquad (1)$$

In the presence of oxygen and formate ions the radicals react to form superoxide anion radicals ($O_2^-$), see Table I. Using the rate constants given in Table I, we

TABLE I

REACTIONS OCCURRING IN OXYGENATED SODIUM FORMATE SOLUTIONS DURING OR AFTER IRRADIATION

| | Reaction | $k$ ($M^{-1} \cdot s^{-1}$) | Reference |
|---|---|---|---|
| 2 | $e^-_{aq} + O_2 \rightarrow O_2^-$ | $(1.9 \pm 0.2) \cdot 10^{10}$ | 1 |
| 3 | $H + O_2 \rightarrow HO_2$ | $2 \cdot 10^{10}$ | 2 |
| 4 | $OH + HCO_2^- \rightarrow H_2O + CO_2^-$ | $2.8 \cdot 10^9$ | 3 |
| 5 | $H + HCO_2^- \rightarrow H_2 + CO_2^-$ | $1.5 \cdot 10^8$ | 2 |
| 6 | $CO_2^- + O_2 \rightarrow CO_2 + O_2^-$ | $2.4 \cdot 10^9$ | 4 |
| 7 | $O_2^- + O_2 \xrightarrow{-2H^+} O_2 + H_2O_2$ | $\sim 10^2$ | 5 |
| 8 | $HO_2 + O_2 \xrightarrow{-2H^+} O_2 + H_2O_2$ | $8.5 \cdot 10^7$ | 5 |
| 9 | $HO_2 + HO_2 \rightarrow O_2 + H_2O_2$ | $7.6 \cdot 10^5$ | 5 |
| 10 | $HO_2 \rightarrow H^+ + O_2^-$ $\quad pK = 4.9 \pm 0.1$ | | 5 |

calculated that at the concentration of oxygen and sodium formate employed in our experiments (see later), reactions 2–6 will finish in less than 1 $\mu$s. If we assume the yield of the combined radical species ($G$) to be 6.5 molecules/100 eV absorbed i.e. $G = 6.5$, and conversion of the radicals quantitative to $O_2^-$, a dose of 150 rad will produce 1 $\mu$M $O_2^-$. At low pH, the superoxide anion disappears rapidly by reacting with the hydrodioxyl radical ($HO_2$) (see reaction 8). To minimize this, we used low doses and a five times higher concentration of ferricytochrome $c$ with respect to those at pH $\geqq$ 7.

It is clear that with the pulse-radiolysis technique $O_2^-$ is easily produced and in high yields. The radical is an oxidizing as well as a reducing agent: $E_0'$ ($O_2^-/H_2O_2$) = +0.94 V and $E_0'$ ($O_2/O_2^-$) = −0.33 V [6] and it is therefore unstable. The superoxide radical is sometimes considered to be harmful to molecules of biological importance, but the cause of the damage is not known (see for review, Bors et al. [7]). These interesting features, together with the fact that the result of earlier determinations of the rate constant of the reaction of $O_2^-$ with ferricytochrome $c$ in our laboratory (Wilting, J., unpublished) did not agree with the literature values [8, 9], led us to investigate in greater detail the kinetics and products of the reaction:

ferricytochrome $c + O_2^- \rightarrow O_2$ + ferrocytochrome $c$ (11)

Independent studies similar to part of those reported here have been carried out by Butler et al. [10] and Simic et al. [11].

## MATERIALS AND METHODS

Horse heart cytochrome $c$ was isolated according to the method of Margoliash and Walasek [12]. The final preparation, chromatographed on Amberlite CG 50 (particle diameter 40–60 $\mu$m) to remove modified material, was deionized exhaustively on mixed-bed columns, and stored at −20 °C. Horse heart cytochrome $c$ was also obtained from Koch-Light (90–100 %) and used without further purification. The concentration of the hemoprotein was determined spectrophotometrically using a $\Delta A_{550\,nm}$ (red-ox) of 21 $mM^{-1} \cdot cm^{-1}$ [13]. Superoxide dismutase, isolated according

to the method of McCord and Fridovich [14] was a gift from Dr. R. Wever (Laboratory of Biochemistry, B. C. P. Jansen Institute, University of Amsterdam).

Sodium formate, analytical grade, was purchased from Fluka AG (Buchs, Switzerland). All other chemicals were of analyzed reagent quality and were obtained from J. T. Baker (Deventer, Netherlands). Deionized water, predistilled under nitrogen from an alkaline permanganate solution was redistilled in an all glass apparatus. The solutions to be irradiated were purged with oxygen (purity: 99.5 %, Hoek-Loos, Amsterdam, Netherlands) for at least half an hour under a pressure of $1.5 \pm 0.1$ atm. The oxygen concentration in equilibrium with this pressure is $2.02 \pm 0.13$ mM [15].

In the pulse-radiolysis experiments the solutions were irradiated with single 2 MeV electron pulses (550 ns duration) from a 2 MV Van de Graaff accelerator (High Voltage Engineering (Europe) N.V., Amersfoort, Netherlands). The cell forms part of a single beam spectrophotometer consisting of a 450 W Xenon lamp, shutter, 2-cm cell, Bausch and Lomb-grating monochromator, 1P 28 photomultiplier and a dual time base Tektronix 7904 oscilloscope fitted with a polaroid camera. The monochromator settings were calibrated against ferrocytochrome *c*. The slit width was 2 nm.

Sometimes the concentration of $O_2^-$ was higher than 10 % of the ferricytochrome *c* concentration, so that the reactions did not follow simple first-order kinetics. Therefore, we magnified the traces from the photographs 2.5 times, and analyzed them routinely according to the formula [16, 17]:

$$\ln \frac{[c_t^{2+}]}{[c_e^{2+}]} - \ln \left[1 - \frac{[O_2^-]}{[c_0^{3+}]} \left(1 - \frac{[c_t^{2+}]}{[c_e^{2+}]}\right)\right] = k_1([c_0^{3+}] - [O_2^-])t \qquad (12)$$

in which $[c_t^{2+}]$ is the concentration of ferrocytochrome *c* at time *t*, $[c_e^{2+}]$ is the final ferrocytochrome *c* concentration, $[c_0^{3+}]$ is the concentration of ferricytochrome *c* at $t = 0$ and $k_1$ is de second-order rate constant. The initial concentration of $O_2^-$ was determined from the increase in net charge of an insulated diaphragm just in front of the cell by using a Keithly 601 electrometer. The charge increments have been standardized by thiocyanate dosimetry [18].

For continuous radiation we used a $^{60}Co$ $\gamma$-source (Gammacell 200, Atomic Energy, Canada). The activity of the cytochrome *c* was measured in the ascorbate-*N,N,N',N'*-tetramethylphenylenediamine-cytochrome *c* oxidase system [19]. Copper was determined by atomic absorption using a Perkin-Elmer 300.

All results are expressed as $\bar{x} \pm (ts/\sqrt{n_d})$ in which $\bar{x}$ is the mean and *s* the standard deviation of $n_d$ determinations; *t* is a factor dependent on $n_d$ and the confidence level. In our studies, $n_d$ varied between 3 and 6, and for the confidence level we chose 95 % [20].

All measurements were carried out at $21 \pm 2$ °C.

## RESULTS

Both cytochrome *c* and $O_2^-$ are charged species and thus a high ionic strength of the matrix solution can interfere with the reaction. We therefore preferred to do the experiments at the lowest possible concentration of sodium formate. To determine a suitable low concentration of formate, we measured the yield of the reduction of ferricytochrome *c* at 550 nm as a function of the formate concentration. This is shown

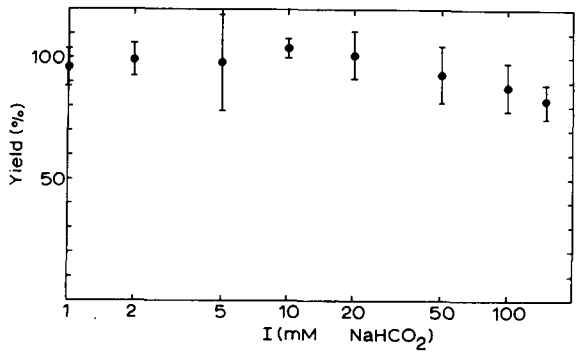


Fig. 1. Effect of the ionic strength on the yield of the reaction of ferricytochrome *c* with $O_2^-$. Conditions: pH 7.1 ± 0.1, 10.5–13.5 μM ferricytochrome *c*; EDTA was present in a molar ratio of 1 to 100 with respect to sodium formate; at high ionic strength, 0.4–0.8 mM phosphate buffer was present; the dose absorbed varied between 140 and 190 rad. See text for explanation of the symbol *I*.

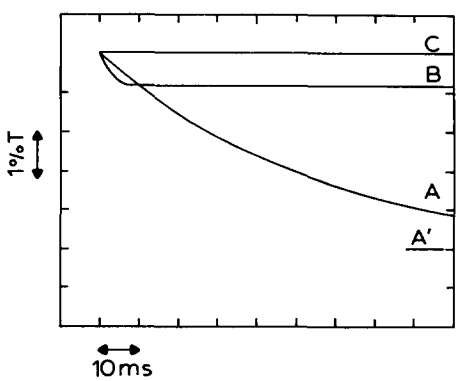


Fig. 2. Oscilloscope traces of the reaction of $O_2^-$ with ferricytochrome *c*. Conditions: 17 μM ferricytochrome *c*; 20 μM EDTA; 2 mM sodium formate and 1 mM phosphate buffer (pH 7.2). Dose absorbed: 145 rad. A, Decrease of transmittance at 550 nm; A', final transmittance. B, Change of transmittance in the presence of 95 nM superoxide dismutase. C, Idem in the presence of 0.95 μM superoxide dismutase.

in Fig. 1. As can be seen, the yield of the reaction is nearly constant between 2 and 50 mM sodium formate and decreases somewhat at higher concentrations. Even at 2 mM sodium formate, the yield is very near the 100 % calculated from the *G* value of 6.5. We therefore used this concentration for our further studies.

The reduction of cytochrome *c* was measured at 550 nm, the main absorbance maximum of ferrocytochrome *c* in the visible region. Fig. 2, trace A, shows the increase in 550 nm absorbance when a solution containing 17 μM ferricytochrome *c*, 1 mM phosphate buffer (pH 7.2), 2 mM $NaHCO_2$, 20 μM EDTA and approx. 2 mM $O_2$ is irradiated with a 0.5 μs pulse of 2.0 MeV electrons (dose absorbed 145 rad).

Under the conditions of measurement, the reduction of ferricytochrome *c* is most probably due to the reaction of the hemoprotein with the superoxide radical $O_2^-$. Since, however, ferricytochrome *c* is also rather reactive towards $e_{aq}^-$, $OH^\cdot$ and

$CO_2^-$, it is just possible that a part of the reduction is caused by direct reaction of these species with cytochrome *c*. To estimate the contribution of such a direct reduction we added small amounts of superoxide dismutase to the matrix. This enzyme catalyses reaction 7 specifically [14]. A typical example of such an experiment is also shown in Fig. 2, trace B. As can be seen the presence of 95 nM superoxide dismutase inhibits the reduction in such a way that only 8 % of the expected absorbance change is observed. Addition of more superoxide dismutase completely abolishes the reduction of the hemoprotein (Fig. 2, trace C); thus it is safe to assume that under the conditions of measurement the reduction of ferricytochrome *c* is solely due to its reaction with $O_2^-$. Since the rate constant for the reduction of ferricytochrome *c* by $O_2^-$ is $1.1 \cdot 10^6\ M^{-1} \cdot s^{-1}$ (see later) the rate constant for the reaction of $O_2^-$ with superoxide dismutase can be calculated from the trace B in Fig. 2 and is $(2.3 \pm 0.2) \cdot 10^9\ M^{-1} \cdot s^{-1}$ ($n_d = 5$) at $I = 4$ mM, pH 7.2 and 20 °C. We consider this a simple competition reaction, because $O_2^-$ reacts equally fast with oxidized and reduced superoxide dismutase [21] and the ferricytochrome *c* concentration does not change much during the reaction. The value found is in good agreement with those values reported in literature: $2.3 \cdot 10^9$–$2.6 \cdot 10^9\ M^{-1} \cdot s^{-1}$ [21–23].

Not only does the protein-bound copper of superoxide dismutase react rapidly with $O_2^-$, but so do copper ions and copper-formate complexes. The second-order rate constant of the reaction of $O_2^-$ with $Cu^{2+}$ is $2.7 \cdot 10^9$–$8 \cdot 10^9\ M^{-1} \cdot s^{-1}$ ($I =$ 1 mM, $t =$ 20–24 °C, pH 7.5) [21, 24]. The copper-formate complexes formed at high concentrations of sodium formate are also very reactive with $O_2^-$: $k = 1 \cdot 10^9\ M^{-1} \cdot s^{-1}$ at 100–150 mM sodium formate in the presence of 1.90–1.85 M $NaClO_4$ [25]. It can be seen from Table II that copper is present in a substantial amount in the matrix solutions containing large concentrations of formate. To keep this reaction from interfering with our further studies we added 20 μM EDTA to our solutions and used water distilled twice from an all glass apparatus. EDTA has no effect on the reduction of ferricytochrome *c* by $O_2^-$ [26].

The reduction of ferricytochrome *c* by $O_2^-$ is first order in cytochrome *c* and to study this reaction in more detail we measured the apparent first-order rate constant ($k_1'$) for the reaction as a function of the concentration of cytochrome *c*. Fig. 3 shows the results. As can be seen the $k_1'$ increases linearly with increasing concentration of cytochrome *c* indicating a virtual absence of intermediates of the reaction. From the slope of the line the second-order rate constant for the reaction ($k_1$) can be calculated to be $(1.1 \pm 0.1) \cdot 10^6\ M^{-1} \cdot s^{-1}$ at $I = 4$ mM, pH 7.2, and 20 °C. Since the line is

TABLE II

DETERMINATION OF COPPER IN VARIOUS SAMPLES

The error is estimated to be 20 %.

| Sample | Concentration | Copper found, in ppb |
|---|---|---|
| Sodium formate | 1.0 M | 30 |
| Cytochrome *c*, our preparation | 1.0 mM | 20 |
| Cytochrome *c*, Boehringer | 1.0 mM | 20 |
| Cytochrome *c*, Sigma, Type VI | 1.0 mM | 25 |
| Water, twice distilled | 55.5 M | 0 |

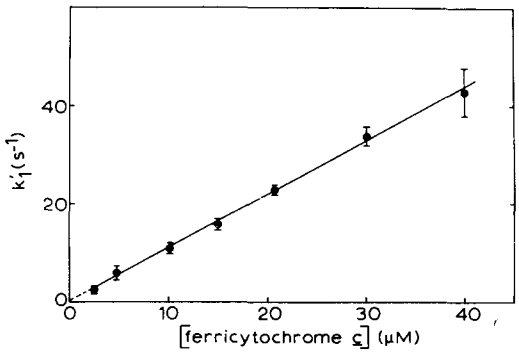


Fig. 3. Dependence of the apparent first-order rate constant on the concentration of ferricytochrome *c*. Conditions: 2 mM sodium formate; 20 $\mu$M EDTA; 1 mM phosphate buffer, pH 7.2. Dose absorbed varied between 100 and 750 rad.

seen to pass the origin it is concluded that the dissociation rate constant ($k_{-1}$) is very small. The reaction is almost stoichiometric and the yield of the reaction is independent of the concentration cytochrome *c* or $O_2^-$ used (not shown). $O_2^-$ can act as an oxidizing agent, we therefore measured the degree of reduction after 10–20 pulses (100–200 % $O_2^-$ with respect to ferricytochrome *c*). Since the amount of cytochrome *c* reduced this way was 90–100 % of that originally present, it is concluded that $O_2^-$ does not oxidize ferrocytochrome *c*.

The conformation of cytochrome $c^{3+}$ depends on the pH of the matrix with transition points at pH 1.2, 3.4 and 9.3 [27]. The conformational change at pH 9.3

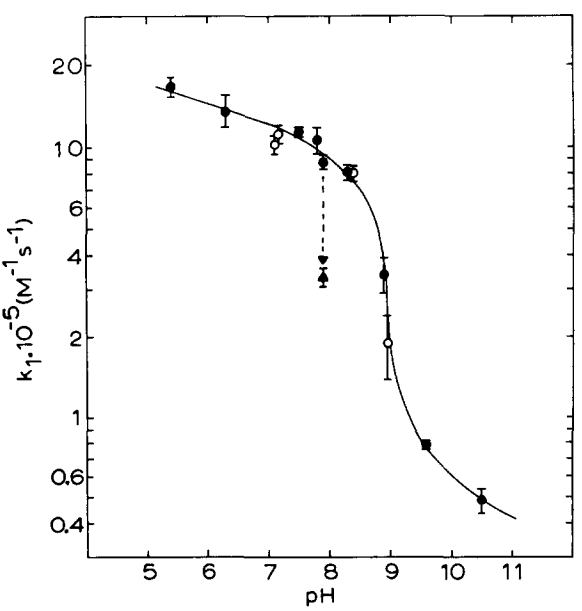


Fig. 4. Dependence of the observed second-order rate constant on the pH. Conditions: 7–25 $\mu$M ferricytochrome *c*, except at pH 5.4 and pH 6.3, where it was 64 and 51 $\mu$M, respectively; 2 mM sodium formate; 1 mM phosphate or borate buffer and 20 $\mu$M EDTA. Dose absorbed: 140$\pm$10 rad (●) or 150–500 rad (○). ▲, 0.10 M sodium formate, 10 mM phosphate buffer and 1 mM EDTA.

causes a dramatic decrease in both the yield and the rate of the reaction of $e_{aq}^-$ with ferricytochrome *c* [28, 29]. Therefore we investigated the effect of the pH on the rate of reaction of $O_2^-$ with ferricytochrome *c* at low ionic strength.

Fig. 4 summarizes the results. Between pH 5.4 and 8 the rate of reaction decreases slightly with increasing pH; at pH > 8, however, the rate of reaction decreases drastically: by more than a factor of 10 between 8.3 and 9.6. Between pH 5.6 and 10.5 a monophasic reduction is observed. When larger doses of radiation were used at pH 8.9 ($[O_2^-] \geqq \frac{1}{2}$[cytochrome $c^{3+}$]) a biphasic reaction was observed, the initial rapid reduction being followed by a slower reduction with a half time of about 2 s.

Simic et al. [11] and Butler et al. [10] observed a fairly sharp decrease in rate constant between pH 7.0 and 7.4. Their measurements, however, were carried out at high ionic strength ($I = 0.15$ and 0.10 M, respectively) and this may have been the reason why their results differ from ours. We therefore determined the second-order rate constant of the reaction of $O_2^-$ with ferricytochrome *c* at "high" and "low" ionic strength. Our results which show a clear effect of the ionic strength on the rate constant at pH 7.9, together with those of Butler et al. [10] and Simic et al. [11], are summarized in Table III. As can be seen there is little discrepancy in the results.

To quantify the effect of ionic strength we measured the second-order rate constant of the reaction of $O_2^-$ with ferricytochrome *c* at several concentrations of sodium formate at pH 7.1. According to the Brønsted equation [17] the observed rate constant is a function of the ionic strength:

$$\log k_{obs} = \log k_{I=0} + \frac{1.02\, Z_A Z_B I^{\frac{1}{2}}}{1+\alpha I^{\frac{1}{2}}} \tag{13}$$

in which $k_{obs}$ is the observed second-order rate constant, $k_{I=0}$ the second-order rate constant at zero ionic strength, $Z_A$ the charge of particle A, $Z_B$ the charge of particle B, $I$ the ionic strength, defined as $I = \frac{1}{2}\Sigma m_i z_i^2$ (with $m$ = molality), and $\alpha$ a constant, depending on the minimum distance between the reacting particles A and B. $\alpha$ was taken equal to 6. The equation predicts a straight-line relationship between log $k_{obs}$

TABLE III

INFLUENCE OF IONIC STRENGTH ON THE SECOND-ORDER RATE CONSTANT OF THE REACTION OF $O_2^-$ WITH FERRICYTOCHROME *c* AT pH 7.9

| Authors | Cytochrome *c* | $10^{-5} \times k$ ($M^{-1} \cdot s^{-1}$) | |
|---|---|---|---|
| | | Low ionic strength[a] | High ionic strength[b] |
| This paper | Our preparation | 8.8±0.5 | 3.4±0.3 |
| This paper | Koch-Light | 7.5±0.2 | 4.0±0.3 |
| Butler et al. [10] | Koch-Light | n.d. | 4.8±0.5 |
| Simic et al. [11] | Sigma, Type VI | n.d. | 5.5±0.5[c] |

[a] 1 mM phosphate buffer, 2 mM sodium formate.
[b] 10 mM phosphate buffer, 100 mM sodium formate.
[c] The conditions of Simic et al. [11] were slightly different: 150 mM sodium formate and 1 mM phosphate buffer.

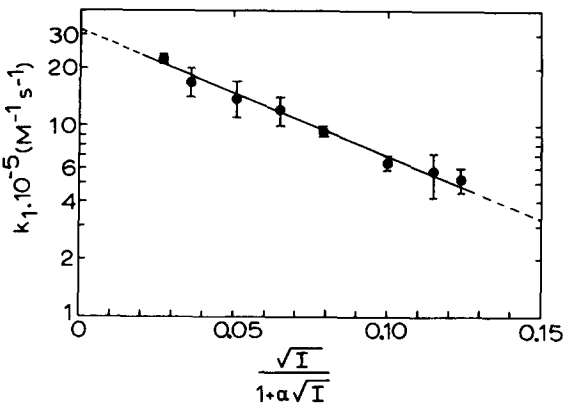


Fig. 5. Dependence of the observed second-order rate constant on the ionic strength. Conditions: see Fig. 1. See text for explanation of the symbols $I$ and $\alpha$.

versus $I^{\frac{1}{2}}(+\alpha I^{\frac{1}{2}})^{-1}$. From the intercept on the abscissa we can determine the value of $k_{I=0}$, and from the slope of the line the value of $Z_A \cdot Z_B$. Our results are shown in Fig. 5. We determined $k_{I=0}$ as $(3.1 \pm 0.1) \cdot 10^6\ M^{-1} \cdot s^{-1}$ and a value of $-6.3$ for $Z_A \cdot Z_B$. As $Z_A$, the charge on the $O_2^-$ radical, is $-1$, the charge on the cytochrome *c* molecule is found to be $+6.3$. If it is assumed that two formate ions are bound per molecule of ferricytochrome *c*, then this charge is in excellent agreement with that obtained by spectrophotometric titration [30].

To find out whether the reaction of $O_2^-$ with cytochrome *c* damages the protein we measured the enzymatic activity in the ascorbate-TMPD-cytochrome *c* oxidase system after irradiation in a $^{60}Co$ $\gamma$-source. Preliminary results showed no appreciable decrease in activity up to doses of radiation absorbed corresponding to 3–4 mol of $O_2^-$ per mol of cytochrome *c*.

## DISCUSSION

From the results presented it is clear that at neutral pH the reaction of $O_2^-$ with ferricytochrome *c* is a straightforward second-order reduction with a yield of $99 \pm 6$ % (2 mM $NaHCO_2$, Fig. 1) in which no detectable intermediates are observed.

Our results obtained at pH 7.9 using Koch-Light cytochrome *c*, are in reasonable agreement with those reported by Butler et al. [10] and Simic et al. [11]. After correction for the effects of ionic strength their rate constants at pH < 8, however, are larger. From the data in Table II it can be calculated that in a solution containing 10 $\mu$M cytochrome *c* and 100 mM sodium formate approx. 50 nM copper are present. Although small, this amount cannot be neglected since copper or copper-formate complexes react, catalytically, at least 2000 times faster with $O_2^-$ than ferricytochrome *c* reacts. Part of the copper may also be bound to cytochrome *c*. As Butler et al. [10] and Simic et al. [11] did not use EDTA we can explain the discrepancy between their results and ours by assuming that the copper present in their solutions caused an overestimation of the rate constants. Above pH 8 their results are more consistent with ours, probably because an inactive copper-hydroxide complex is formed.

From Fig. 3 it was concluded that the reverse reaction:

ferrocytochrome $c + O_2 \rightarrow$ ferricytochrome $c + O_2^-$ (14)

is slow with respect to the forward reaction. This is in agreement with the recent observations of Sawada et al. [31] who reported that the second-order rate constant for reaction 14 is approx. $3 \cdot 10^{-2}$ $M^{-1} \cdot s^{-1}$ at pH 7.0, $I = 100$ mM and 25 °C. With this rate constant and the one for the forward reaction reported here ($k_1 = 0.57 \cdot 10^6$ $M^{-1} \cdot s^{-1}$) the change in free energy for the reduction of ferricytochrome $c$ under these conditions can be calculated from $\Delta G_0' = RT \ln k_1/k_{-1}$ in which $R$ is the gas constant and $T$ the absolute temperature. The value of $\Delta G_0'$ is 10.0 kcal/mol $O_2^-$.

Oxygen can exist in energetically different states, of which the triplet ground state, symbol $^3\Sigma_g^-$, and two reactive singlet forms, designated as $^1\Delta_g$ and $^1\Sigma_g^+$ are of importance. If one of the singlet states is the product of the reaction of $O_2^-$ with proteins like cytochrome $c$, this may be very harmful for the protein itself or other biomolecules in the direct vicinity [39, 40]. From an oxidation-state diagram of oxygen, adapted from Phillips and Williams [32, 33] (Fig. 6), one can, however, conclude that the formation of singlet oxygen is unlikely. In this diagram the free energy at pH 7, $\Delta G_0'$, of several oxygen compounds is plotted against the formal charge on the oxygen atom. The free energy is related to the reduction potential through the relation $\Delta G_0' = nF\,\Delta E_0'$. Therefore, the reduction potential of a certain couple can be obtained from such a diagram by measuring the slope of the line joining

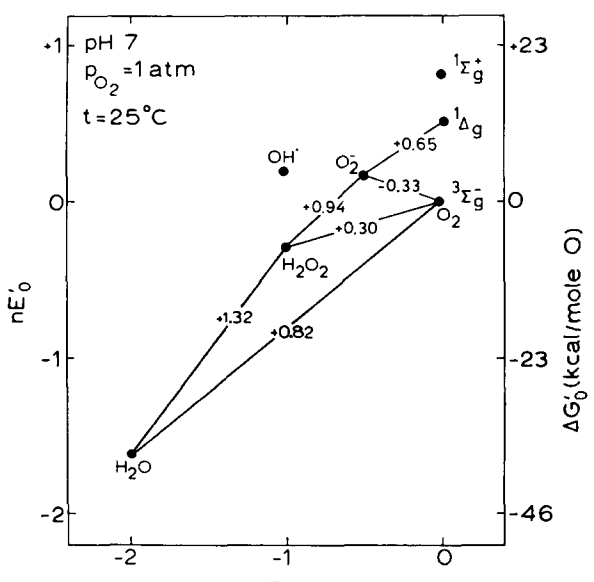


Fig. 6. Oxidation-state diagram of oxygen at pH 7. Abscissa: formal charge $n$ per oxygen atom; ordinate, left: $n$ times reduction potential ($E_0'$); ordinate, right: free energy per oxygen atom at pH 7 ($\Delta G_0'$). The numbers refer to the slopes of the line. $E_0'$ (A/B) denotes the reduction potential of the couple A/B at pH 7 under otherwise standard conditions, relative to the potential of the normal hydrogen electrode. For the construction of this diagram, data from refs. 6, 36 and 37 were used. The differences in entropy between the several forms of oxygen have been neglected in view of the uncertainties in the values of the energy differences.

the members of the couple. Thus the reduction potential of the couple $^1\Delta_g O_2/O_2^-$ is +0.65 V, whereas that of the couple $^3\Sigma_g^- O_2/O_2^-$ is −0.33 V, etc. From these considerations it is clear that the reduction potential of the couple ferri-/ferrocytochrome *c*, being +0.26 V [30], is too low to oxidize $O_2^-$ to any form of singlet oxygen. One can also conclude that no singlet oxygen is formed in view of the fact that the change in free energy for the reaction in which triplet oxygen is formed can be calculated via $\Delta G_0' = nF\,\Delta E_0'$ to be −13.6 kcal, in good agreement with the value derived from $\Delta G_0' = RT \ln k_1/k_{-1}$, −10.0 kcal. In line with these conclusions is the observation that the cytochrome *c* molecule is not damaged by its reaction with $O_2^-$.

The haem iron of cytochrome *c* is buried deep inside the protein molecule. The question as to how reducing equivalents are transported from the surface of the molecule to the $Fe^{3+}$ has not yet been answered. At present three hypothetical mechanisms exist [27]: (1) The reductant binds to the surface of the cytochrome *c* molecule at or in the vicinity of Tyr-74 and an electron is transported through the protein moiety via aromatic residues; (2) the reductant binds to the exposed haem edge and the electron is transported via the porphyrin ring to the haem iron; or (3) the haem iron is reduced by a quantum mechanical process, called electron tunnelling. Of these hypotheses the first has been ruled out because Tyr-74, which plays an important role in that mechanism, is replaced by leucine in bacterial cytochrome *c*-550 [27] or Tyr-74 can be iodated without loss of enzymatic activity [34]. The second hypothesis is attractive for several reasons. From experiments with a water-soluble cobalt (III)-porphyrin it has been shown that the reduction by $Cr^{2+}$ occurs via the porphyrin $\pi$-electron cloud [38]. The reaction of $O_2^-$ with cytochrome *c* is slow compared with that of the hydrated electron; thus it is likely that $O_2^-$ reacts at a specific site. The reaction is influenced by the ionic strength, indicating that electrostatic effects play a role, in agreement with the observation that the haem edge is surrounded by a cluster of positive charges [27]. Furthermore, it is unlikely that $O_2^-$ is able to reduce an amino acid as $e_{aq}^-$ can, but it may react directly with the haem since the ferri/ferro haem couple in cytochrome *c* has a reduction potential 0.59 V (pH 7) higher than the $O_2/O_2^-$ couple. The conformational change around pH 9.3, induced by the replacement of the sulphur atom of Met-80 by the nitrogen atom of probably Lys-79, is accompanied by a decrease in reduction potential. It is probably this which causes the unreactivity of cytochrome *c* with respect to $O_2^-$ at high pH values. The rate of reduction also decreases with increasing pH, and since the p*K* of 9.0, estimated from the pH curve (Fig. 4), agrees very well with the p*K* reported by Margalit and Schejter [30] for the dependence of the reduction potential on the pH, we believe that the pH curve reflects the conformational change. If this is the case, the slow change in transmission observed at pH 7.9 using large doses of radiation is a measure of the rate of transition of the inactive high-pH conformation to the active low-pH form. The rate constant for this process (designated as C → A in the scheme of Lambeth et al. [35]) is approx. 0.3 $s^{-1}$.

Reduction of the haem may also be brought about by an electron which tunnels from the surface of the protein to the $Fe^{3+}$. The small but distinct amount of activation energy of 20 kJ observed by Butler et al. [10], however, points towards a more conventional reduction mechanism.

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