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Relation between Redox Potentials and Rate Constants in Reactions Coupled with the System Oxygen–Superoxide†

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ABSTRACT: Univalent oxidation–reduction reactions coupled with the oxygen–superoxide system were investigated in the reactions shown in eq 3 and 8, where Q and $Q\cdot^-$ stand for *p*-benzoquinone and *p*-benzosemiquinone, respectively. From kinetic experiments the following rate constants were obtained at pH 7.0: $k_3 = 4.5 \times 10^4\ M^{-1}\ sec^{-1}$ and $k_8 = 3 \times 10^{-2}\ M^{-1}\ sec^{-1}$. With known values of k_{-3} and k_{-8}, and of E_0' for the systems Q–$Q\cdot^-$ (0.10 V) and Cyt c^{3+}–Cyt c^{2+} (0.255 V), the calculated values of $E_0(O_2$–$O_2\cdot^-)$ were found to lie in the range between −0.27 and −0.33 V.

The formation of superoxide radical was suggested in reactions of xanthine oxidase (Fridovich and Handler, 1958) and of peroxidase–oxidase (Yamazaki and Piette, 1963). Accumulation of the radical during the above reactions has been confirmed by means of electron spin resonance (ESR) spectroscopy (Knowles et al., 1969; Nilsson et al., 1969). Since the previously known copper proteins, erythrocuprein and hemocuprein, were found to have a catalytic function accelerating dismutation of superoxide anion radicals (McCord and Fridovich, 1969) a large number of papers have been devoted to the study of the formation and reactivity of the superoxide radical. Stepwise oxidation, as formulated by Michaelis (1951), becomes of fundamental significance in the kinetic analysis of reactions involving the superoxide radical. Experimental evidence which shows the relation between one-electron redox potentials in a bivalent system and rate constants has been reported by Yamazaki and Ohnishi (1966).

A reliable value for the O_2–$O_2\cdot^-$ redox potential is needed in order to analyze reactions coupled with its redox system. By thermodynamic calculation the redox potential was reported as being −0.56 V by Latimer (1952). The similar-

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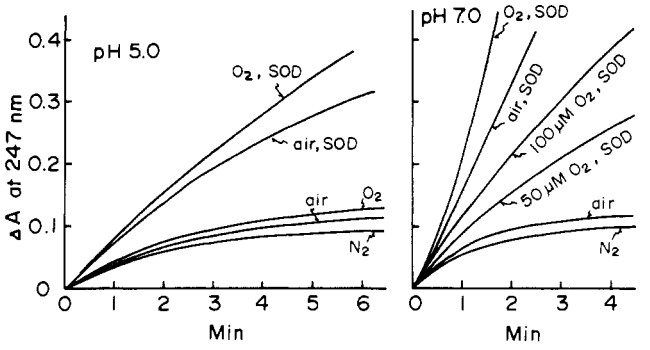

FIGURE 1: Time courses of *p*-benzoquinone formation through peroxidase reaction under various conditions. The reaction mixture contained 0.2 m*M* hydroquinone, 5 μ*M* H_2O_2, and 0.6 n*M* horseradish peroxidase. The experiments were carried out at pH 5.0 (left) and 7.0 (right) under various O_2 concentrations (indicated in the figure). The concentration of superoxide dismutase (SOD) was 2 μ*M*. The reaction was started by the addition of H_2O_2.

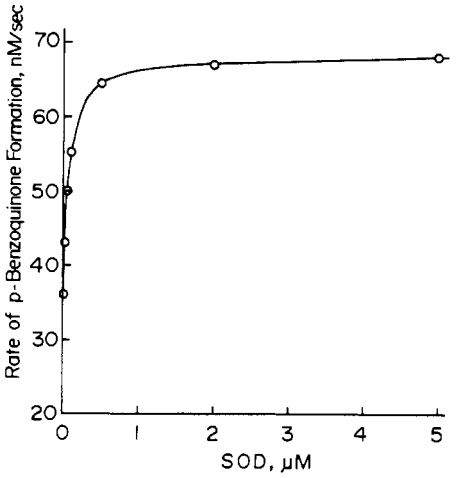

FIGURE 2: Effect of the concentration of superoxide dismutase (SOD) on the initial rate of *p*-benzoquinone formation. The reaction solution was air saturated and the pH was 5.0. The other experimental conditions was as described in Figure 1.

ly calculated value by George (1965) was −0.59 V as the correction was made by Sutin (1965). However, these values appeared to be too low to explain the formation and reactivity of superoxide radicals (Yamazaki et al., 1965). Wood (1974), summarizing recent data, has concluded that the value of −0.33 V is most reliable for the redox potential. The same value has been reported independently by Ilan et al. (1974).

In this paper we shall report kinetic analyses of redox reactions coupled with the O_2–O_2scnpt$^-$ system and the relation between redox potentials and rate constants will be discussed.

Materials and Methods

Horseradish peroxidase was purified from wild horseradish roots by the method of Shannon et al. (1966) with slight modification. The enzyme preparation used was a mixture of peroxidase isoenzymes B and C. Superoxide dismutase was prepared from dry seeds of *Pisum sativum* (Sawada et al., 1972). Horse heart cytochrome *c* was obtained from Boehringer. All other materials were obtained from commercial sources at the highest available states of purity.

Absorbance measurements were carried out with a Hitachi recording spectrophotometer, Model 124, equipped with

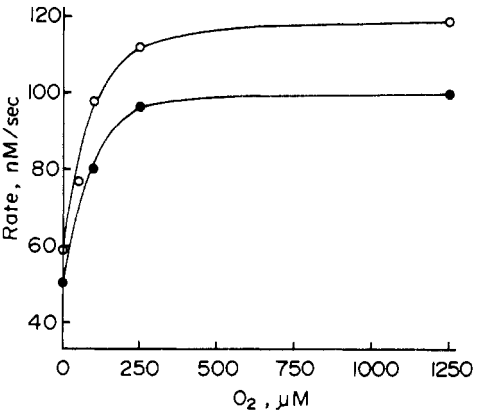

FIGURE 3: Effect of the concentration of O_2 on the initial rate of *p*-benzoquinone formation. The reaction mixture contained 2 μ*M* superoxide dismutase: pH 5.0 (●) and pH 7.0 (○). The other experimental condition was as described in Figure 1. Solid lines are calculated from eq 4. The values used are: $k_d = 8 \times 10^7\ M^{-1}\ sec^{-1}$, $k_3 = 4.5 \times 10^4\ M^{-1}\ sec^{-1}$, $V_m = 101$ n*M* sec^{-1} (pH 5.0), and $V_m = 119$ n*M* sec^{-1} (pH 7.0). [Q·$^-$] was calculated from eq 5.

a thermostatically controlled cell compartment. Reactions were performed at 25° in 0.05 *M* buffer solutions of sodium acetate (pH 5.0) and potassium phosphate (pH 7.0). The concentration of O_2 in reaction solutions was controlled by bubbling N_2, O_2, or a mixture of both. Highly purified N_2 gas (99.9995%) obtained from a commercial source was used to maintain anaerobic conditions.

Results and Kinetics

Reaction of p-Benzoquinone–p-Benzosemiquinone Coupled with the System O_2–O_2·$^-$. Hydroquinone (H_2Q) undergoes one-electron oxidation forming *p*-benzosemiquinone through peroxidase reaction (Yamazaki et al., 1960):

$$2H_2Q + H_2O_2 \xrightarrow{\text{peroxidase}} 2Q\cdot^- + 2H_2O + 2H^+ \quad (1)$$

The value of pK_a for *p*-benzosemiquinone has been reported to be 4.25 (Yamazaki and Piette, 1965). As the reaction of peroxidase with the semiquinone is negligible under ordinary reaction conditions (Yamazaki, 1971), under aerobic conditions the semiquinone disproportionates nonenzymatically to *p*-benzoquinone and hydroquinone:

$$2Q\cdot^- (+ 2H^+) \xrightarrow{k_d} Q + H_2Q \quad (2)$$

or reduces O_2 to form the superoxide radical:

$$Q\cdot^- + O_2 \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} Q + O_2\cdot^- \quad (3)$$

The rate constant k_3 was previously assumed to be negligibly small because hydroquinone could not serve as a hydrogen donor in the peroxidase-oxidase reaction (Yamazaki, 1958). As the rate constant k_{-3} has been measured to be $10^9\ M^{-1}\ sec^{-1}$ by Patel and Willson (1973) and $9.8 \times 10^8\ M^{-1}\ sec^{-1}$ by Rao and Hayon (1973) it appears that the reaction of *p*-benzosemiquinone with O_2 is not appreciable only because its back reaction is much faster. By careful stoichiometric experiments the amount of *p*-benzoquinone formed in the peroxidase reaction consuming a limited amount of H_2O_2 was found to exceed slightly the molar amount of H_2O_2 under aerobic conditions as shown in Figure 1. It was concluded that the extra amount of hydroquinone was oxidized with consumption of O_2. Figure 1 alo shows that the O_2-consuming oxidation of hydroquinone

Table I: Effects of the Concentration of O_2 and Ferricytochrome c on the Rate of Autoxidation of Ferrocytochrome c at pH 7.0.

$[O_2]$ (mM)[a]	Rate of Oxidation (10^{-10} M sec^{-1})
0.25	2.3
1.25	8.5
[Ferricytochrome c] (μM)[b]	Rate of Oxidation (10^{-10} M sec^{-1})
1.5	6.0
9.7	4.4
18	2.7
34	1.3

[a] 30 μM ferrocytochrome c (containing about 7% ferricytochrome c). [b] 18.5 μM ferrocytochrome c and 1.25 mM O_2.

was markedly increased in the presence of superoxide dismutase, which might eliminate the back reaction in eq 3. The reaction of H_2O_2 with superoxide dismutase (Hodgson and Fridovich, 1973; Bray et al., 1974) could be neglected under these conditions. The time-dependent acceleration of the rate at pH 7.0 in the presence of superoxide dismutase and saturated O_2 could be explained according to Yamazaki and Ohnishi (1966).

The initial rate of p-benzoquinone formation increased up to a constant level as the concentration of superoxide dismutase was increased (Figure 2). The addition of p-benzoquinone, as is expected from eq 3, retarded the O_2-consuming oxidation of hydroquinone. It is shown in Figure 3 that in the presence of a sufficient amount of superoxide dismutase the initial rate of p-benzoquinone formation was increased with an increase of O_2 concentration and reached almost twice as much as that measured under anaerobic conditions. The maximum rate V_m could be regarded as the rate of p-benzosemiquinone formation. In the presence of superoxide dismutase the rate of p-benzoquinone formation (V) can be formulated as the following equation provided that no chain reaction takes place:

$$d[Q]/dt = V = k_d[Q\cdot^-]^2 + k_3[Q\cdot^-][O_2] \quad (4)$$

The rate of p-benzosemiquinone formation (V_m) should equal the rate of its decay at steady state:

$$V_m = 2k_d[Q\cdot^-]^2 + k_3[Q\cdot^-][O_2] \quad (5)$$

Then:

$$V_m - V = k_d[Q\cdot^-]^2 \quad (6)$$

$$2V - V_m = k_3[Q\cdot^-][O_2] \quad (7)$$

The rate constant k_d has been reported to be 7×10^7 M^{-1} sec^{-1} by Diebler et al. (1961) and 8.0×10^7 M^{-1} sec^{-1} by Yamazaki and Ohnishi (1966). From eq 6 and 7 and data in Figure 3 the value of the rate constant k_3 could be calculated as 4.5×10^4 M^{-1} sec^{-1} at pH 5.0 and 7.0.

Reaction of Cytochrome c-Reduced Cytochrome c Coupled with the System O_2-$O_2\cdot^-$. The reduction of cytochrome c (Cyt c) by the superoxide radical has been used for simple confirmation of superoxide formation and for a measure of superoxide dismutase activity (McCord and Fridovich, 1969; Fridovich, 1972). Recently the rate constant for the reaction of cytochrome c with the superoxide radical has been measured by pulse radiolysis (Land and Swallow, 1971) and kinetic analysis (Massey et al., 1973; Sawada and Yamazaki, 1973). On the contrary, though reduced cytochrome c is slowly autoxidized under aerobic conditions, the rate has been considered to be too slow to give a reliable value for k_8;

Table II: Rate Constants; k_8 (ferrocytochrome c + O_2) and k_{-8} (ferricytochrome c + $O_2\cdot^-$).

	pH	Rate Constant (M^{-1} sec^{-1})	Reference
k_8	7.0	3×10^{-2}	This paper
k_{-8}	7.0	2.5×10^4	Sawada and Yamazaki (1973)
	8.5	2.1×10^4	Sawada and Yamazaki (1973)
	8.4	1.6×10^5	Massey et al. (1973)
	8.5	1.1×10^5	Land and Swallow (1971)
k_{-8}/k_8 at pH 7.0 = 8×10^5 (3.7×10^6)[a]			

[a] The value in parentheses was obtained on the assumption that $k_{-8} = 1.1 \times 10^5$ M^{-1} sec^{-1} at pH 7.0.

$$\text{Cyt } c^{2+} + O_2 \underset{k_{-8}}{\overset{k_8}{\rightleftharpoons}} \text{Cyt } c^{3+} + O_2\cdot^- \quad (8)$$

In Table I it is shown that the rate of oxidation of the cytochrome was approximately proportional to the concentration of O_2 and was decreased with an increase of oxidized cytochrome c. The value of k_8 could then be roughly estimated at 3×10^{-2} M^{-1} sec^{-1} when the concentration of oxidized cytochrome c was extrapolated to 0. Superoxide dismutase accelerated the oxidation of reduced cytochrome c but the enzyme could not be used for kinetic analysis of reaction 8 probably because the reaction was too slow to eliminate nonspecific side reactions such as a direct redox reaction between cytochrome c and superoxide dismutase. Dependent upon experimental methods the value of k_{-8} varied over a wide range and only approximate values of the equilibrium constant of reaction 8 could be obtained (Table II).

Discussion

From kinetic analyses of an equilibrium between p-benzoquinone-p-benzosemiquinone and cytochrome c-reduced cytochrome c systems the following relation has been experimentally confirmed by Yamazaki and Ohnishi (1966):

$$E_0(Q\text{-}Q\cdot^-) - E_0'(\text{Cyt } c^{3+}\text{-Cyt } c^{2+}) = -(RT/F) \ln (k_r/k_0) \quad (9)$$

where k_r and k_0 are rate constants in eq 10:

$$Q\cdot^- + \text{Cyt } c^{3+} \underset{k_0}{\overset{k_r}{\rightleftharpoons}} Q + \text{Cyt } c^{2+} \quad (10)$$

There is very little doubt that eq 9 is applicable to a reaction composed of an elementary reaction in either a forward or backward direction. Similarly, it might be possible to measure the redox potential E_0* of the O_2-$O_2\cdot^-$ system according to the following equations:

$$E_0^* - E_0(Q\text{-}Q\cdot^-) = -(RT/F) \ln (k_{-3}/k_3) \quad (11)$$

$$E_0^* - E_0'(\text{Cyt } c^{3+}\text{-Cyt } c^{2+}) = -(RT/F) \ln (k_{-8}/k_8) \quad (12)$$

where E_0* was calculated on the basis of the molar concentration of O_2. Then, $E_0(O_2\text{-}O_2\cdot^-) = E_0^* - 0.17$ V (Wood, 1974). The results thus obtained are listed in Table III. These values are in good agreement with the data obtained by others using equilibrium methods.

Recently developed techniques of pulse radiolysis have made it possible to measure the equilibrium constant of reaction 3 in the case of duroquinone (Patel and Willson,

Table III: Values of $E_0(O_2\text{-}O_2\cdot^-)$ Based on Experimental Measurements.

E_0(V)	Method and Reference
−0.270	Polarography, Chevalet et al. (1972)
−0.33	Equilibrium, Berdnikov and Zhuravleva (1972)
−0.33	Pulse radiolysis and rapid equilibrium, Wood (1974); calculated from Patel and Willson (1973), Bishop and Tong (1965), and Rao and Hayon (1973)
−0.33	Pulse radiolysis and rapid equilibrium, Ilan et al. (1974)
−0.33	Eq 11: $k_3 = 4.5 \times 10^4 M^{-1}$ sec^{-1}, $k_{-3} = 9.8 \times 10^8 M^{-1}$ sec^{-1} (Rao and Hayon, 1973); $E_0(Q/Q\cdot^-) = 0.10$ V (Yamazaki and Ohnishi, 1966)
−0.27 (−0.31)	Eq 12: Table II, E_0' of cytochrome c at pH 7.0 = 0.255 V

1973; Ilan et al., 1974). Wood (1974), using the data of Patel and Willson (1973) and Bishop and Tong (1965), has calculated the value of $E_0(O_2\text{-}O_2\cdot^-)$ to be −0.33 V. This value is the same as that reported independently by Ilan et al. (1974). Rao and Hayon (1973; 1974a,b) have also reported the redox potentials of various free radicals including the superoxide radical. However, as is pointed out by Wood (1974), there is a serious error in their deduction of the redox potential. Although the observed equilibrium after pulse radiolysis is considered to be that of the reaction $R\cdot^- + A \rightleftharpoons R + A\cdot^-$, the redox potential of the system $R\text{-}R\cdot^-$ is calculated using values of $E_0'(A\text{-}AH_2)$. For their calculation $E_0(A\text{-}A\cdot^-)$ should be used instead. Rao and Hayon (1973) reported the value of $E_0(O_2\text{-}O_2\cdot^-)$ to be +0.15 V. Based on the data of Rao and Hayon (1973) and of Bishop and Tong (1965), Wood (1974) has corrected the value to −0.33 V. It has been reported by Greenwood and Palmer (1965) that there are two functionally distinct forms of cytochrome c monomer at alkaline pH. Brandt et al. (1966) on the oxidation-reduction reactions between ferricyanide and cytochrome c have shown that at alkaline pH the equilibrium constant deviates from the ratio of rate constants. At these pH values the assumption that reaction 10 is composed of one elementary reaction in either a forward or backward direction will not be valid. If consideration is given to these points, redox potentials of one-electron transfer systems will be useful parameters in the kinetic analysis of oxidation-reduction reactions. Such studies on the reaction between cytochrome b_5 and oxygen will be reported elsewhere.

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