Biochimica et Biophysica Acta, 440 (1976) 573–586

BBA 47154

REACTIONS OF THE FERRI-FERROCYTOCHROME-*c* SYSTEM WITH SUPEROXIDE/OXYGEN AND CO_2^-/CO_2 STUDIED BY FAST PULSE RADIOLYSIS

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(Received January 23rd, 1976)

SUMMARY

The reduction of ferricytochrome *c* by O_2^- and CO_2^- was studied in the pH range 6.6–9.2 and Arrhenius as well as Eyring parameters were derived from the rate constants and their temperature dependence. Ionic effects on the rate indicate that the redox process proceeds through a multiply-positively charged interaction site on cytochrome *c*. It is shown that the reaction with O_2^- (and correspondingly with O_2 of ferrocytochrome *c*) is by a factor of approx. 10^3 slower than warranted by factors such as redox potential. Evidence is adduced to support the view that this slowness is connected with the role of water in the interaction between O_2^-/O_2 and ferri-ferrocytochrome *c* in the positively charged interaction site on cytochrome *c* in which water molecules are specifically involved in maintaining the local structure of cytochrome *c* and participate in the process of electron equivalent transfer.

INTRODUCTION

The reactions of the ferri-ferrocytochrome *c* (CIII-CII) system with many reducing and oxidizing agents have been studied [1]. Of considerable biochemical and biological interest is its reaction with oxygen and with its one-electron equivalent reduction product, the radical O_2^- (at neutral or alkaline pH) or HO_2 (at acid pH). The autoxidation reaction was studied under various conditions [2–5] and found to yield O_2^- in a slow reaction. The slowness of the oxidation of CII by O_2 is very important from the point of view of its task in vivo, since it should react with cytochrome *c* oxidase but not with O_2, which is present in its environment.

The reduction of CIII by the superoxide anion radical O_2^-:

$$\text{CIII}+O_2^- \rightarrow \text{CII}+O_2 \qquad (1)$$

which is the back reaction of the autoxidation, was studied less extensively. Using the technique of pulse radiolysis, Land and Swallow [6] found that CIII is reduced

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by O_2^- at basic pH (8.5) with a rate constant of $1.1 \times 10^5 \cdot M^{-1} \cdot s^{-1}$. Simic et al. [7] studied the dependence of the reduction rate on pH. The results of these two pulse radiolytic studies are in good agreement with the results of Ballou et al. [8] who studied the same reaction by the technique of rapid-freezing/electron-paramagnetic-resonance.

The relationship between redox potentials and rate constants in reactions coupled with the system oxygen-superoxide, including those with CIII/CII, was discussed by Sawada et al. [5]. For the CIII/CII system itself to obtain meaningful comparisons in this respect, one should compare O_2^-/O_2 with reagent having similar charge type and size, since in reactions with CIII/CII not only the redox potential of the reagent but also its mode of interaction with CIII/CII, determined by charge type and structural factors, will affect reaction rates.

The reactions of the couple CO_2/CO_2^- may also be readily studied by the technique of pulse radiolysis. It is of a similar charge type as well as not greatly different in size, as O_2/O_2^-, but greatly differs in its redox potential.

The CO_2^- anion radical is known to reduce CIII much faster than does O_2^- [6]:

$$CO_2^- + \text{CIII} \xrightarrow{k_2} \text{CII} + CO_2 \tag{2}$$

with $k_2 \approx 5\text{–}10 \times 10^9 \cdot M^{-1} \cdot s^{-1}$.

Using a pulse radiolytic apparatus we undertook a study of the reduction of CIII by O_2^-, and compared it with its reduction by CO_2^-. Our aim was to gain better understanding of the reasons for the relative slowness of the reactions of cytochrome *c* with O_2 and with O_2^-.

EXPERIMENTAL

Materials. Sigma type III or type VI horse heart CIII was used without further purification. The results were similar with both types. The proportion of the ferroenzyme was less than 7 % as determined by absorption at 550 nm. The concentration of CIII was determined by the 528 nm absorption peak, using $\varepsilon = 11.2 \cdot 10^3 \cdot M^{-1} \cdot cm^{-1}$ [9]. The water used was distilled first from alkaline permanganate, then from phosphoric acid, and finally redistilled.

Argon, N_2O and CO_2 were supplied by Matheson. When needed, these gases were freed of oxygen by bubbling through a solution containing V(II) prepared by in situ reduction of Fluka purum grade $NaVO_3$ with zinc amalgam prepared from B.D.H. analytical reagent zinc and Frutarom analytical grade mercury. Oxygen was supplied by the Israel Oxygen Centre.

t-Butanol (Merck, pro analysi) contained no impurities detectable by ultraviolet absorption spectrometry. Sodium formate was supplied by B.D.H., sodium sulfate, $NaH_2PO_4 \cdot H_2O$ and $Na_2H\ PO_4$ by Mallinckrodt, sodium hydroxide by Riedle De Haen. All reagents were of analytical grade, and were used without further purification. The ethanol used was Fluka fur U.V.-Spektroskopie.

Apparatus. The Varian linear accelerator of the Hebrew University and the optical and electronic systems are described elsewhere [10]. The irradiation cells were rectangular flow-through cells fabricated of high purity silica. Their optical paths were either 2 or 4 cm. For temperature effect studies, we used a rectangular cell of 3 cm optical path, surrounded by a bigger cell through which water from a thermostated water bath was circulated. Three optical windows of the cell were left free of the thermostated water for entry of the electron beam and for passing the analytical light through the cell.

Spectra of solutions were taken on a Cary 14 spectrophotometer, and pH measurements on a digital pHM52 of Radiometer, or on a pH-meter of El-Hama Instruments, Israel.

The temperature within the cell was calibrated using a thermocouple and was found to be equal to that of the water bath within ±0.5 °C.

Procedures. The pH of the solutions was adjusted by adding 2 mM phosphate buffer for pH < 8, or by adding NaOH, above pH 8.

Solutions were prepared in glass bottles no more than 10 h before use. Solutions were kept after preparation at the stated pH for approx. 1 h before using them for irradiation, so that equilibrium could be reached.

Deaeration of solutions and saturation with N_2O, O_2 or CO_2 were accomplished by sweeping with argon or with the gas in question for at least 15 min. Solutions were saturated with gas and irradiated in large glass syringes equipped with capillary standard taper joints. Irradiations were carried out no more than 0.5 h after sweeping with gas.

The absorbed dose per pulse was determined routinely using the spectrum of hydrated electrons produced by pulsing $1 \cdot 10^{-2}$ M aqueous ethanol at pH 9.5–10, taking $\varepsilon_{578\,\mathrm{nm}} = 1.06 \cdot 10^4 \cdot \mathrm{M}^{-1} \cdot \mathrm{cm}^{-1}$, and $G(e_{aq}^-) = 2.75$ molecules/100 eV [11].

Doses used ranged from 100 to 1500 rad per pulse (equivalent to $3 \cdot 10^{-7}$–$4 \cdot 10^{-6}$ M of e_{aq}^-). To assure pseudo first order conditions the concentrations of O_2^- or CO_2^- were approx. 10 times lower than those of CIII. When unthermostated irradiation cells were used, the temperature was 22±1 °C.

Oscilloscope traces were analyzed by transferring the data to punched cards by means of a magnifying manual trace follower coupled to an analogue to digital converter, and processing the cards in a Control Data Corp. Cyber digital computer. The kinetics was followed by recording the change in absorption at 450 and at 550 nm. The rate constants reported in this paper are subject to an error of ±15 %. Energies of activation and related derived parameters (cf. Figs. 1 and 2) have an error of less than ±10 %.

RESULTS

The reduction of CIII by O_2^- was studied using solutions containing HCO_2Na and O_2. Formate scavenges OH radicals, and leads to additional O_2^- formation. The following reactions occur in the pulse-irradiated solution.

$$H_2O \rightarrow e_{aq}^-,\ H,\ OH,\ H_2,\ H_2O_2 \qquad (3)$$

$$e_{aq}^- + O_2 \rightarrow O_2^- \quad k_4 = 2 \cdot 10^{10} \cdot M^{-1} \cdot s^{-1} \quad [12] \qquad (4)$$

$$H + O_2 \rightarrow HO_2 \quad k_5 = 2 \cdot 10^{10} \cdot M^{-1} \cdot s^{-1} \quad [13] \qquad (5)$$

$$H + HCO_2^- \rightarrow H_2 + CO_2^- \quad k_6 = 5 \cdot 10^8 \cdot M^{-1} \cdot s^{-1} \quad [14] \qquad (6)$$

$$OH + HCO_2^- \rightarrow H_2O + CO_2^- \quad k_7 = 2.7 \cdot 10^9 \cdot M^{-1} \cdot s^{-1} \quad [15] \qquad (7)$$

$$CO_2^- + O_2 \rightarrow O_2^- + CO_2 \quad k_8 = 4.2 \cdot 10^9 \cdot M^{-1} \cdot s^{-1} \quad [16] \qquad (8)$$

As a consequence of these reactions, the only reagents present shortly after the pulse, and able to react with CIII, are O_2^- and HO_2. The concentrations of HCO_2^- and of O_2 were always high enough so as to scavenge all e_{aq}^-, H and OH radicals generated by the pulse.

O_2^- and HO_2 are in equilibrium with a pK_a of 4.88 [17]. Thus, at pH > 6, most of the radicals are converted to O_2^-, and its concentration is given by the sum: $[e_{aq}^-]+[OH]+[H] = 2.2[e_{aq}^-]$ [18].

The ratio: $[O_2]/[CIII]$ was always greater than 25, so that less than 1 % of the CO_2^- radicals react with CIII directly. Since the reduction of CIII by O_2^- is relatively slow, it has to compete with the decay of O_2^- by Reactions 9–11 [17]:

$$HO_2+HO_2 \longrightarrow H_2O_2+O_2 \quad k_9 = 7.6 \cdot 10^5 \cdot M^{-1} \cdot s^{-1} \tag{9}$$

$$HO_2+O_2^- \xrightarrow{H_2O} H_2O_2+OH^-+O_2 \quad k_{10} = 8.5 \cdot 10^7 \cdot M^{-1} \cdot s^{-1} \tag{10}$$

$$O_2^-+O_2^- \xrightarrow{2H_2O} H_2O_2+O_2+2\,OH^- \quad k_{11} < 100 \cdot M^{-1} \cdot s^{-1} \tag{11}$$

Of these, Reaction 10 is the most significant in the pH range of this work. Since the concentration of HO_2 is low, the extent of Reaction 9 is small, and so is that of Reaction 11, with its low rate constant.

In order to avoid this competition, we kept the ratio $[CIII]/[O^-_2]$ higher than 30 when the pH was lower than 7, by using high [CIII] and a low dose. At higher pH, the competition by Reaction 10 also becomes negligible because of the very low concentrations of HO_2.

The reduction of CIII at concentrations in the range of $5 \cdot 10^{-6}$–$5 \cdot 10^{-5}$ M in oxygen or air saturated solutions was found to follow pseudo first order kinetics, the rate being proportional to [CIII]. The results at four pH values are summarized in Table I together with the relevant values from other works. From the observed reduction yield together with the difference spectrum before and after the pulse which was compared with the spectrum calculated from ref. 9, we conclude that CIII is quantitatively reduced by O_2^-, to produce CII. A similar result was found for the reduction of CIII by CO_2^- [6].

When studying the reduction of CIII by CO_2^-, the solutions were saturated with N_2O (≈ 25 mM). This scavenges all the electrons and converts them to OH radicals according to Reaction 12:

TABLE I

SECOND ORDER RATE CONSTANTS FOR THE REDUCTION OF CIII BY O_2^-

	Temperature	pH			
		6.6	7.3	≈ 8.5	9.2
This work	20 °C	$6.2 \cdot 10^5$	$5.0 \cdot 10^5$	$2.6 \cdot 10^5$*	$2.0 \cdot 10^5$
Ref. 7	Ambient	$1.5 \cdot 10^6$	$9.0 \cdot 10^5$	$2.5 \cdot 10^5$*	$2.0 \cdot 10^5$
Ref. 6	Ambient	—	—	$1.1 \cdot 10^5$**	—
Ref. 8	20 °C	—	—	$1.6 \cdot 10^5$***	—

* pH = 8.7
** pH = 8.5
*** pH = 8.4

$N_2O + e_{aq}^- \rightarrow N_2 + OH^- + OH \cdot \; k_{12} = 5.6 \cdot 10^9 \cdot M^{-1} \cdot s^{-1}$ [12] (12)

The OH and H radicals react with the formate anions according to Reactions 6 and 7, producing CO_2^- anion radicals. Alternatively, (when studying ionic strength effects at neutral pH) the solutions were saturated with CO_2 which reacts with hydrated electrons according to Reaction 13:

$CO_2 + e_{aq}^- \rightarrow CO_2^- \; k_{13} = 7.7 \cdot 10^9 \cdot M^{-1} \cdot s^{-1}$ [12] (13)

The OH radicals were scavenged in this case by *t*-butanol according to Reaction 14:

$(CH_3)_3COH + OH \rightarrow H_2O + \cdot CH_2C(CH_3)_2(OH)$

$k_{14} = 6.3 \cdot 10^8 \cdot M^{-1} \cdot s^{-1}$ [15] (14)

The concentration of *t*-butanol was 10^4-times higher than that of CIII, assuring that less than 1 % of the OH radicals react with CIII directly. The rate of CIII reduction by both O_2^- and CO_2^- increased with increasing temperature. The plots of log *k* versus $1/T$ at various pH are given in Figs 1 and 2. From these plots, Arrhenius activation energies were derived. The values of activation energies are summarized in Table II.

A decrease in reduction rates was observed as the ionic strength was increased (by adding Na_2SO_4), as expected for a reaction between two oppositely charged species. The plots of log k versus the square root of ionic strength yield almost straight lines which are shown in Figs. 3 and 4. From the slopes of these lines effective charges were calculated. These are given in Table III. Complex formation between

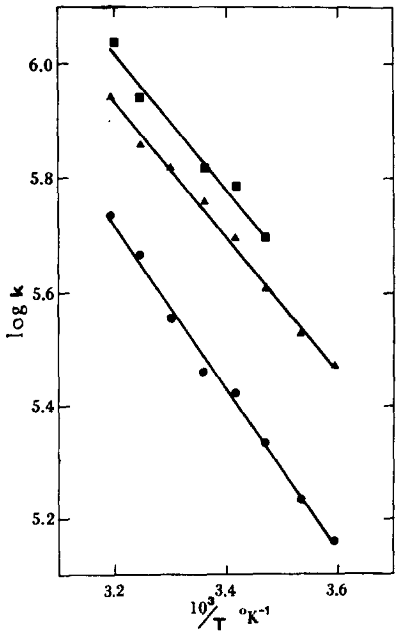

Fig. 1. Derivation of Arrhenius activation energies for the reduction of CIII by O_2^-. $\mu = 0.1$ (NaHCOO). ●, pH 8.7; ▲, pH 7.3; ■, pH 6.6.

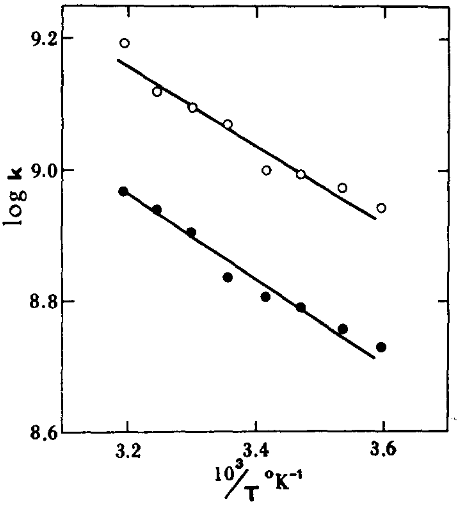

Fig. 2. Derivation of Arrhenius activation energies for the reduction of CIII by CO_2^-. $\mu = 0.1$. (NaHCOO). ●, pH 8.7; ○, pH 6.2.

CIII and O_2^- was not detected, either by spectral changes in the visible and in the ultraviolet parts of the spectrum, or by an influence on the kinetics of CII formation or of O_2^- decay, which we followed by measuring its absorption at 260 nm.

TABLE II

(a) Arrhenius activation energies E_a kcal/mol for the reduction of CIII by O_2^- and by CO_2^- (μ = 0.1 (0.1 M NaHCOO)) and pre-exponential factors A calculated from the rate constant k and the values of E_a.

Reducing ion	pH	E_a	k (20 °C)	A
O_2^-	6.6	5.4	$6.2 \cdot 10^5$	$6.2 \cdot 10^9$
	7.3	5.4	$5.0 \cdot 10^5$	$5.0 \cdot 10^9$
	8.7	6.6	$2.6 \cdot 10^5$	$2.0 \cdot 10^{10}$
CO_2^-	6.2	2.7	$1.0 \cdot 10^9$	$1.0 \cdot 10^{11}$
	8.7	3.0	$6.3 \cdot 10^8$	$1.1 \cdot 10^{11}$

(b) Eyring parameters for transition state calculated from plot of log (k/T) vs. $1/T$ (data as in (a) above), using the value of $2 \cdot 10^{10}$ for (αR/hN)

	pH	$\Delta S^{\neq}$ cal/degree per mol	$\Delta H^{\neq}$ kcal/mol
O_2^-	6.6	—14.5	5.2
	7.3	−15.5	5.0
	8.7	−13.4	5.9
CO_2^-	6.2	−8.9	2.4
	8.7	−9.5	2.5

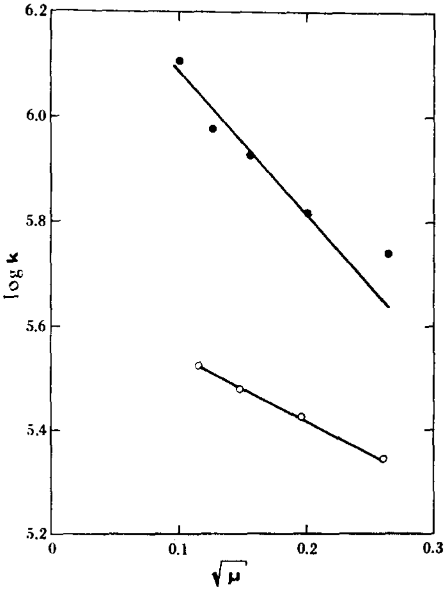

Fig. 3. Log *k* versus the square root of the ionic strength for the reduction of CIII by O_2^-. Ionic strength changed by adding Na_2SO_4. [NaHCOO] = 0.005 M. ○, pH 8.7; ●, pH 6.6.

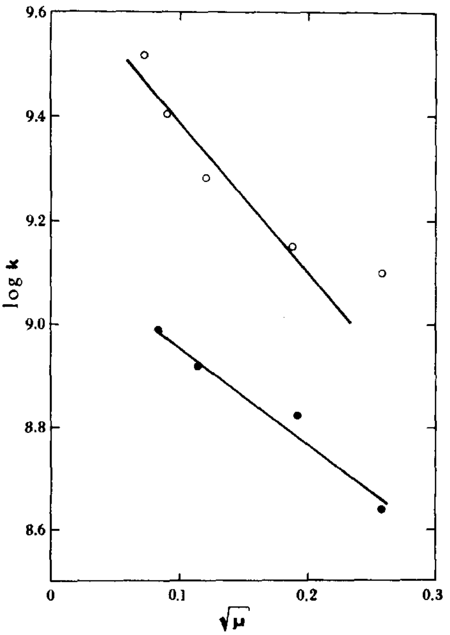

Fig. 4. Log *k* versus the square root of the ionic strength for the reduction of CIII by CO_2^-. Ionic strength changed by adding Na_2SO_4. [NaHCOO] = 0.005 M. ●, pH 8.7; ○, pH 6.6.

TABLE III

EFFECTIVE CHARGES FOR THE REDUCTION OF CIII BY O_2^- AND BY CO_2^-

Ionic strength changed by adding Na_2SO_4.

Reducting ion	pH 6.6	pH 8.7
O_2^-	+2.8	+1.3
CO_2^-	+2.8	+1.9

The rate of reduction of CIII by O_2^- decreased at neutral pH from $6 \cdot 10^5 \cdot M^{-1} \cdot s^{-1}$, in the presence of 0.1 M formate, to $3.8 \cdot 10^5 \cdot M^{-1} \cdot s^{-1}$ in the presence of 0.05 M $NaClO_4$ and 0.05 M formate and thus the same ionic strength of 0.1.

DISCUSSION

The reaction of ferrocytochrome *c* with molecular oxygen is of biological interest. The slowness of this reaction assures that CII is not autoxidized significantly in competition with its proper biochemical pathway of reaction with cytochrome *c* oxidase. Relation with the redox potentials of the partners was sought to interpret the observed rates [5]. We have to remember, however, that the potential *E* is related to the ratio of the rate constants of the forward and back reactions, k_1/k_2 and is thus consistent with a very wide number of individual rate constants, provided only that the faster process does not exceed the limit of diffusion controlled rate. One would like to have a better understanding of the molecular reasons for the magnitude of the specific rate constants. In this, we can benefit from the recent results on the interaction between redox reagents of various types and CII/CIII [19, 20] and resulting rate constants, as well as a comparison between the O_2^-/O_2 system and the CO_2^-/CO_2 system, which differ in redox potential but are rather similar in some molecular properties, such as charge type and ion size.

(*a*) *pH effect on the reduction by* O_2^-

The rate constants obtained in this work are compared with the results of Simic et al. [7] in Table I. At basic pH they are in excellent agreement, and higher than those in refs. 6 and 8. At neutral and slightly acid pH, our constants are about one half of those presented in ref. 7. One should bear in mind that at neutral and acid pH the decay of O_2^- occurs by its reaction with HO_2 (Reaction 10) and by the reduction of CIII by O_2^- (Reaction 1).

When competition by Reaction 10 is not negligible, the reduction yield is lower than 100 %, kinetics become faster and deviate from apparent first order. To obtain accurate values for the rate constant of Reaction 1 at neutral and acid pH high concentrations of CIII, and low concentrations of $HO_2(O_2^-)$, i.e. a low dose, has to be used. When $[CIII]/[O_2^-] \leqslant 10$, at neutral pH, we observed a decrease in CIII reduction yield and an apparent increase in the reduction rate. Our results in Table I at $[CIII]/[O_2^-] \approx 30$, are for reduction yields > 90 %. Exact comparison of results from different sources has to take into account the temperatures of solutions.

As shown by Simic et al. [7], the rate constant decreases with increasing pH. In the pH range 6.6–9.2 which we cover in the present work, the redox potential of CII/CIII does not change appreciably [21] nor are there considerable spectroscopic changes observed in CII or CIII. The observed pH effect on rate may be due to some structural change, e.g. in an interaction site, which does not affect spectrum or redox potential significantly, but does so to the molecular details of the kinetics, in both directions. We observed a pK_a of 7.4 in the cytochrome *c* molecule, in the kinetics of oxidation of the nonrelaxed ferroenzyme by ferricyanide in the presence of perchlorate [19]. In the present case, a protonation-deprotonation process with pK_a in this range, the reduction of the protonated molecule being faster than that of the deprotonated molecule may account for the facts.

The decrease of the rate constant with increasing pH may in this way also be connected with the change in activation energy with pH, as will be discussed below.

(b) Comparison of the reduction of CIII by O_2^- and by CO_2^-

The Arrhenius activation energy for the reduction of CIII by CO_2^- (Table II) is low and compatible with activation energies for diffusion controlled reactions. Similar values were observed for the reduction of CIII by some organic radicals [22]. The Arrhenius energy of activation of the reduction of CIII by O_2^- (Table II) is by 2.5–3.5 kcal/mol higher than the activation energy of its reduction by CO_2^-. Such a difference would cause the reduction by CO_2^- to be $\approx 10^2$ times faster than the reduction by O_2^- if preexponential factors were similar. The experimental ratio of rate constants is more than 10^3 ($k_{CO_2^- + CIII} = 5 \cdot 10^8$–$10^9$ at pH < 7, 20 °C, from Fig. 2 and refs. (6 and 7), so that an additional difference in factors is required. The redox potential $E^{0\prime}$ (for 1 atm O_2) of the couple O_2^-/O_2 is -0.33 V. For 1 M O_2 at pH 7, [23–25], $E^{0\star} = -0.16$ V. The exact redox potential of the couple CO_2^-/CO_2 is not known, but an approximate value from polarographic experiments is ≈ -1.3 V [26]. Due to the more negative redox potential of CO_2^- than that of O_2^-, CO_2^- reduces O_2 very fast in aqueous solutions [16]. The known values of the gas phase electron affinities are $+$ 0.44 eV for O_2 [27] and -0.6 eV for CO_2 [28].

From the rate of reduction of CIII by O_2^-, observed at neutral pH (Table I), combined with the known difference in the redox potential of the couples O_2^-/O_2 [23–25] and CIII/CII [1, 21], we calculate the autoxidation rate constant of CII, $\approx 4.5 \cdot 10^{-2} \cdot M^{-1} \cdot s^{-1}$, which is similar to the experimental value of $\approx 3 \cdot 10^{-2} \cdot M^{-1} \cdot s^{-1}$ [5]. This agreement is consistent with the assumption that the mechanism of the reaction between cytochrome c and oxygen does not involve the formation of a definite complex. This is supported by our extensive experiments aimed at observing such a complex; these gave no indication of its formation.

The small but significant decrease in reduction rate constant with increasing pH now observed for CO_2^- (Table II) is similar to previous observations [6]. The change in activation energy on changing the pH in this case is within the limits of our experimental error. In the reduction of CIII by O_2^-, a significant increase in activation energy of 1.2 kcal/mol is observed on changing the pH from neutral to 8.7. This is enough to slow down the reaction by a factor of ≈ 8 at room temperature. The observed ratio of rate constants at room temperature is ≈ 2.5. Thus another factor partly compensates the effect of the greater activation energy. Such a preexponential factor is calculated in Table II.

The reason for the lower reaction rate at basic pH is not connected with a change in the redox potential of CIII between pH 6.5 and 8.7 [21].

(*c*) *Ionic strength effects*

The effective charge on CIII involved in the interaction with O_2^- and CO_2^- and listed in Table II were derived according to the Bronsted-Debye formula in the range of ionic strengths where the theory no longer rigorously applies. The values of Table III should be regarded as lower limits of the true values.

All the values are positive and greater than 1. This indicates a cluster of positive charges in the reaction site. Positive clusters were found on cytochrome *c* in X-ray diffraction studies [1], and were suggested to be involved in its reduction by Fe $(EDTA)_2^{2-}$ [29] and in its oxidation by $Fe(CN)_6^{3-}$ [19]. In both cases, similar values were observed for the effective charge. A much lower value (+0.4) was derived for the oxidation of CII by Co $(Phen)_3^{3+}$ [30] i.e. a positively charged ion.

It was suggested that the redox reactions of cytochrome *c* with many inorganic ions take place at a common site, close to the exposed edge of the porphyrin ring [29–31]. This is also supported by the X-ray diffraction studies of the molecule [1] which show a positive cluster composed of lysines near this edge of the heme. Our results are consistent with the reduction of CIII by O_2^- and CO_2^- taking place at such a site. Because of the close approach of these relatively small reagents they will be affected by the local charge of the reactive site and not by that of the entire macromolecule.

The smaller effective charges observed at basic pH indicate either a deprotonation reaction of one of the groups in the reaction site, or a conformational change which causes a decrease in the number of charges in the reaction site.

The possibility of a deprotonation is in accord with the suggestion of a pK_a of 7–8 on cytochrome *c* as derived from experiments on its reactions with iron-hexacyanide [19], in the presence of ClO_4^-. Such a deprotonation could explain the change of rate and of activation energy with the change in pH.

The effect of ClO_4^- in decreasing the reduction rate is indeed similar to the effect of perchlorate on the rate of oxidation of CII by $Fe(CN)_6^{3-}$ [19]. There it was suggested that the effect of ClO_4^- is due to its properties as a water structure breaking ion. This was supported by the correlation made by Aviram [32] between the partial molar entropy of several aqueous anions (Cl^-, Br^-, NO_3^-, SCN^-, ClO_4^-) and their influence on the absorption spectrum of CIII at acid pH. The same reasoning may be applied to explain the present results.

(*d*) *Effects of water structure*

The rate of autoxidation of CII was found to be dependent on the concentrations of alcohol present in the solution [3]. The rate decreased or was steady up to alcohol concentrations of $\approx$ 5 mol % (equal to a mol fraction of 0.05) and increased at higher alcohol concentrations. It was suggested that this effect is connected with a change in conformation of CII induced by the alcohol which caused an exposure of the heme. Alcohol is known to influence the structure of water [33–34]. A dependence on the alcohol concentration was observed: the thermodynamic properties of water/alcohol mixtures show an extremum at a mol fraction of $\approx$ 0.03. It was suggested [33–34] that alcohol stabilises the structure of water up to mol fraction of $\approx$ 0.03, while at higher concentrations alchool causes a decrease in the structure of water. We suggest that the effect of alcohol on the autoxidation rate of CII is due to its effect on water structure, and thus indirectly on CII conformation. The change in water structure in its neighbourhood influences the conformation of the protein in direct contact with the solvent.

(*e*) *Some molecular aspects of the reaction of* O_2^-/O_2 *with CIII/CII*

The preceding experimental observations and other data in the literature lead us to the conclusion that the reduction of CIII by O_2^- (and, correspondingly, the autoxidation of CII) is slower by a factor of $\approx 10^3$ than warranted by a number of relevant experimental parameters.

The standard free energy change (difference in redox potential) in this reaction would be consistent with higher rates. For cytochrome *c* Land and Swallow [20] showed that pyridinyl radicals of different structures with redox potentials varying from -0.35 to -1.1 V reduce CIII with rate constants within the small range of $\approx$ $2\text{–}8 \cdot 10^8 \cdot M^{-1} \cdot s^{-1}$. Cytochrome c_1 a component of cytochrome-reductase, reduces CIII with $k = 3.3 \cdot 10^6$, in spite of its bulk, steric fitting requirements and very small difference in redox potential ($E_{c_1}{}^{0\prime} = +0.225$ V) [35]. Similarly for reduction by O_2^-, Meisel [36] shows that the rates are within a quite narrow range around $3 \cdot 10^8 \cdot M^{-1} \cdot s^{-1}$ with some acceptors varying widely in $E^{0\prime}$, including $E_{\text{acc}}{}^{0\prime} = E_{O_2^-}{}^{0\prime}$. Thus the reason is likely to lie in a specific mechanistic aspect of the interaction between CIII and O_2^-, in the transition state effective in the electron transfer, when molecular configuration and interatomic distances for reagents and products lead to a minimum in the excess free energy required to complete the process [37].*

The high rates of the reactions of O_2^- with other reagents and the rapid reduction of O_2, e.g. by CO_2^-, also exclude spin restrictions specific to O_2^-/O_2 as a major general factor.

The results suggest to us an interpretation which is not uniquely proven, but is consistent with all the observations. It involves the selective participation of water molecules in the transition state interaction between CIII/CII and redox reagents reacting with it at specific interaction site(s). Some indications of this were discussed above in connection with the effects of perchlorate ion on water structure, and hence on the rate of reduction of CIII and the effect of alcohol on autoxidation. At high alcohol concentration the autoxidation accelerates greatly due possibly to conformational changes in the CII macromolecule which is held, in the presence of water, in a specific configuration. Kihara and McCray [38] in experiments on organized cellular components showed that water is required for and is involved in the redox reactions of CIII, e.g. its reduction by reductase, as manifested also by an $H/^2H$ isotope effect.

The ionic effects in the present work indicate that CO_2^- and O_2^- interact with reaction sites on CIII similar to those for ferro-ferri-cyanide, exhibiting effective positive charges, > 1. In the work on ferro-ferri-cyanide [19] we obtained evidence for water participation in the interaction with the binding site(s) and showed definite complex formation with CIII/II, in agreement with the previous work of Stellwagen et

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* Ref. 37, cf. pp. 9–10 for requirement of (ligand and) solvent reorganization to form the activated complex in which the electronic energy of the reactants' activated complex equals that of the products' activated complex.

al. [39]. The interaction sites probably contain clusters of several lysine residues and are situated somewhat remote from the heme itself. Redox processes proceed through the site(s) [19, 39]. In contrast to ferro-ferri-cyanide, neither CO_2^- nor O_2^- shows definite complex formation with CIII, in their interaction with the positive cluster site(s). We have previously shown in our work on ferro-ferri-cyanide that demonstrable complex formation is not an absolute requirement for redox reactions via interaction sites on CIII/CII.

Some indication of the absence of complex formation in electron transfer to $O_{2\,aq}$ is shown in spectroscopic experiments to determine the electron affinity of O_2 in aqueous solution [40]. These yielded an apparent electron affinity of zero and were interpreted as indicating that $O_{2\,aq}$ is incapable of close interaction (in this case with I_{aq}^-) and forms only a contact charge transfer. In the short time scale of the spectroscopic experiments the vibrational rearrangement of O_2 may have been of greater importance than in our kinetic experiments.

Since CO_2^- and O_2^- both react through similar reaction site(s) as ferro-ferri-cyanide as indicated by the ionic effects, why should CO_2^- show a rate $\approx 10^3$ greater than O_2^- if we do not consider the difference in redox potential alone as the sufficient decisive factor, but at most as one component. Consider the structure of $CO_{2\,aq}^-$ and of $O_{2\,aq}^-$. CO_2, linear in the gas phase, does not bind an electron. Hydrated $CO_{2\,aq}^-$ in solution with its high reduction potential has a bent structure and will in a molecular model be rather similar to the hydrated electron, the excess central charge being held mainly by oriented dipolar forces. By contrast, electron binding by O_2 in the gas phase is appreciable but necessitates vibrational activation of the molecule and hydrated $O_{2\,aq}^-$ has an ellipsoidal structure with charge divided over both ends. Water molecules interact with it accordingly bi-locally. This point was briefly considered by Marcus [41] in connection with electron transfer involving O_2^-/O_2. We suggest the possibility that this introduces in the reaction with CIII an additional requirement of water ordering, due to the intimate molecular configuration of water molecules and local protein conformation in the transition state.

The data in Table II give the Arrhenius and also the transition state (Eyring) parameters for the reactions of CIII with CO_2^- and O_2^-. The decisive factor for the relative slowness of the reaction of O_2^- compared with CO_2^- lies in the additional E_a and $\Delta H^{\neq}$ of approximately 3 kcal/mol. We also see that in the transition state with O_2^- somewhat greater ordering is apparent; $\Delta S^{\neq}$ is more negative by some 5 cal/degree per mol than for CO_2^-. CO_2^- in its behaviour belongs to the large group of reagents, collated in Table I of Simic et al. [7] which react with CIII with rates of the order of $10^8 \cdot M^{-1} \cdot s^{-1}$. This indicates that the requirements of CIII itself towards the transition state are not inherently limiting the possible rate of electron transfer. In Table XVII of Dickerson and Timkovich [1] data are collated for a group of inorganic complex reagents, which react with CIII with rates of the order of $10^4 \cdot M^{-1} \cdot s^{-1}$. $O_{2\,aq}^-$ gives results closer to this latter group. The requirement for additional water molecule involvement for O_2^- could be consistent with this. We are not aware of a quantitative treatment of such a problem. A somewhat related, though significantly distinct problem: simultaneous proton transfer along a linear water polymer, has been actively discussed recently [42]. Here the requirement of an additional water molecule resulted in an increase in activation energy. In our model a linear array is not necessarily involved but rather the requirement for $O_{2\,aq}^-$, of say, two water molecules on two apices of the ellipsoid being concertedly involved with O_2^- and the local structure of CIII in the electron transfer step in the transition state.

We note the results obtained for the reactivity of SO_2^- with CIII, compared with that of dithionite ($^-O_2S\text{-}SO_2^-$) [43]. SO_2^- gave a rate constant of $\approx 10^8$ and dithionite $\approx 10^5 \cdot M^{-1} \cdot s^{-1}$. Only a detailed comparison of mechanisms could show whether in fact related structural factors play some role in the similar rates.

In conclusion, the relative slowness of the reaction of O_2^-/O_2 with CIII/CII is suggested to be due to the specific requirements of the interaction of O_2^- in the transition state with a reaction site on CIII. This interaction involves and depends on water molecules and local macromolecular structure in the transition state, in which electron transfer occurs linked to proton movement involving water [38].

NOTE ADDED IN PROOF (Received July 20th, 1976)

Butler et al. [44] have postulated the p$K \cong 7.4$, as we do, here and in previous work [19]. The agreement on this and other points with the present work strengthens the arguments in favor.

ACKNOWLEDGEMENTS

This research was supported under Contract No. NO.E(11-1) 3009 by the U.S. ERDA. Hiroshi Seki (permanent address, the Institute of Physical and Chemical Research, Wako, Saitama 351, Japan) thanks the Israel Government for a Japan/Israel Cultural Exchange Postdoctoral Fellowship.

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