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DO COPPER IONS INFLUENCE THE REDUCTION OF FERRICYTOCHROME *C* BY O_2^-?

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Summary

Recently, it was suggested that the measured rate of reduction of ferricytochrome *C* by O_2^- below pH 8, was too high in the presence of high concentrations of formate (Koppenol, W.H., Van Buuren, K.J.H., Butler J. and Braams, R. (1976) Biochim. Biophys. Acta 449, 157—168).

The high values were attributed to the presence of impurities of copper, which compete for O_2^-. This assumption is consistent with either a decrease in the reduction yield of ferricytochrome *C* in the presence of copper, or with a very fast reaction of Cu(I) with ferricytochrome *C*.

It was previously shown by us and by others that the reduction yield of ferricytochrome *C* by O_2^- is 100%. We measured the rate of reduction of ferricytochrome *C* by Cu(I), and found that this reaction is slow: $k = (1.5 \pm 0.5) \cdot 10^3\ M^{-1} \cdot s^{-1}$.

Therefore, our results rule out the possibility that below pH 8 copper impurities affect the measured rate constant of the reduction of ferricytochrome *C* by O_2^-.

The reduction of ferricytochrome *C* by the superoxide radical (O_2^-):

$$O_2^- + \text{ferricytochrome } C \rightarrow O_2 + \text{ferrocytochrome } C \qquad (1)$$

was recently the subject of several investigations [1—4]. In these studies [1—4], a pH dependence of k_1 in the pH range 6.5—10 was observed. The pH dependence was suggested by Butler et al. to be due to two pK values of cytochrome *C*, one of 7.45 and the second of 9.2 [2]. Koppenol et al. [4] attributed the pH dependence below pH 8 to a competition for O_2^- between cytochrome *C* (Reaction 1) and free or complexed Cu(II) which was assumed to catalyze the dismutation of O_2^-. The most probable catalysis mechanism is described by Reactions 2 and 3 as suggested by Klug-Roth and Rabani [5].

$$O_2^- + Cu(II) \rightarrow O_2 + Cu(I) \tag{2}$$

$$O_2^- + Cu(I) \xrightarrow{2H^+} H_2O_2 + Cu(II) \tag{3}$$

It was suggested that copper is present as an impurity in formate and in cytochrome *C* [4]. The concentration of copper in a solution containing 100 mM formate and 10 μM ferricytochrome *C* was found to be 50 nM [4].

Assuming that the copper complexes do in fact compete for O_2^-, then, if one ignores Reactions 2 and 3 at these concentrations of copper·formate complexes below pH 8, the measured value of k_1 will turn out to be too high. Above pH 8, copper·hydroxide complexes dominate. They are not reactive toward O_2^-, and therefore the presence of copper would not affect the kinetics of Reaction 1 above pH 8.

Klug-Roth and Rabani [5] showed the catalytic effect of copper ions and copper complexes, including copper·formate complexes on the dismutation of O_2^-. They found that the apparent rate of Reactions 2 and 3 in the presence of copper·formate complexes was proportional to the concentration of copper ions. The apparent second order rate constant was: $1.4 \cdot 10^9$ $M^{-1} \cdot s^{-1}$. Their experimental conditions were very similar to those employed in the studies of Reaction 1 [1—3]. The fact that copper·formate complexes have a catalytic effect implies that k_3 cannot be much smaller than k_2.

A significant competition of Reactions 2 and 3 with Reaction 1 would decrease the reduction yield of ferricytochrome *C* simultaneously with its effect on the kinetics of Reaction 1.

If, however, Reaction 4 is faster than Reaction 3, the reduction yield of ferricytochrome *C* would not be affected by the presence of the copper complexes:

$$Cu(I) + \text{ferricytochrome } C \rightarrow Cu(II) + \text{ferrocytochrome } C \tag{4}$$

The net effect will still be a catalysis of O_2^- dismutation, but the reduction yield of ferricytochrome *C* will remain high (approx. 100%). In this case, k_1 measured ignoring Reactions 2 and 4 would also be too high, this again, if one assumes that copper complexes do compete for O_2^-.

In order to check this possibility, we measured k_4. Cu(I) was produced in situ using the pulse-radiolytic technique, in N_2O saturated solutions, containing 20 μM ferricytochrome *C*, 100 mM HCOONa, 2 mM $CuSO_4$ (pH 6.7). The details of the experimental methods were described before [1].

Under these conditions, all the primary radicals produced by the pulse are transformed into CO_2^- radicals. The CO_2^- radicals may react with either Cu(II), or ferricytochrome *C*:

$$CO_2^- + Cu(II) \rightarrow CO_2 + Cu(I) \tag{5}$$

$$CO_2^- + \text{ferricytochrome } C \rightarrow CO_2 + \text{ferrocytochrome } C \tag{6}$$

In order to calculate quantitatively the amount of CO_2^- radicals reacting with Cu(II) in competition with ferricytochrome *C*, we determined k_5.

We followed the decay of the absorbance of CO_2^- radicals at 260 nm, in N_2O saturated solutions containing 50—200 μM $CuSO_4$, 100 mM HCOONa, and 2 mM phosphate buffer (pH 6.8). We used low doses (approx. 200 rad) in order to avoid competition with the second order decay of CO_2^- ($2k=9\cdot 10^8$ $M^{-1}\cdot s^{-1}$ [6]). Under these conditions, the half-life of CO_2^- was proportional to the concentration of Cu(II), $k_5=(1.5\pm 0.3)\cdot 10^8$ $M^{-1}\cdot s^{-1}$.

In order to measure k_4, we used solutions with [Cu(II)] = 2 mM, and [ferricytochrome *C*] = 20 μM. Under these conditions, $k_5\cdot$[Cu(II)] = $3\cdot 10^5$ s^{-1}, and $k_6\cdot$[ferricytochrome *C*] = $2\cdot 10^4$ s^{-1} [1], thus about 94% of CO_2^- reduced Cu(II).

About 10-μs after the pulse, Reaction 5 was completed, and then we followed Reaction 4 at 550 nm (the reduction of ferricytochrome *C*). The results indicated a very slow reaction, which took several minutes to be finished.

We chose [ferricytochrome *C*]$_0$ to be roughly equal to $[CO_2^-]_0$ being formed during the pulse. As CO_2^- almost quantitatively reduces Cu(II) to Cu(I), in our experiments, $[Cu(I)]_0 \approx$ [ferricytochrome *C*]$_0$ = 20 μM.

Plots of $1/(A_t - A_\infty)$ vs. time yielded straight lines, from which $k_5=(1.5\pm 0.5)\cdot 10^3$ $M^{-1}\cdot s^{-1}$ was calculated. (A_t is the absorbance at time t. A_∞ is the absorbance after 500 seconds).

From these results, it is obvious that Cu(I) cannot compete efficiently with ferricytochrome *C* for O_2^-. Therefore, if Cu(II) competes with ferricytochrome *C* for O_2^-, a decrease in reduction yield is expected, in contradiction to the experimental results.

We conclude that copper ions do not influence the kinetics of the reduction of ferricytochrome *C* by O_2^-. Therefore, the rapid decrease of the second order rate constant observed in refs. 1—3 in the pH range 6.5—8, is real and not an artifact arising from the contamination of solutions by copper ions, as suggested by Koppenol et al. [4]. Koppenol et al. [4] also observed a pH dependence of the rate constant in the pH range 6.5—8, but the change in the rate constant observed by them was much smaller than that observed by the other workers [1—3].

A reason for the difference in the pH dependence observed by the different groups might be the different ionic strength of solutions used. In ref. 4, the ionic strength was 0.005, whereas in the other studies it was 0.1.

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