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CONSECUTIVE OXIDATION AND REDUCTION OF CYTOCHROME *c* IN THE PRESENCE OF HYDROGEN PEROXIDE AND COPPER HISTIDINE

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SUMMARY

Copper (bis) histidine (CuHis) catalysed the oxidation of ferrocytochrome *c* (cyt. c^{2+}) by H_2O_2. At pH 8.7 the rate of oxidation of the ferrocytochrome *c* was negligible unless both the H_2O_2 and the copper were present. The reaction was first order with respect to time in the presence of excess H_2O_2, while true kinetic dependence of the reaction was found to be

$$\frac{d\,(\text{cyt. } c^{2+})}{dt} = k\,(\text{cyt. } c^{2+})\,(\text{CuHis})\,(H_2O_2)$$

with a third order rate constant equal to $5 \cdot 10^4$ $l^2 \cdot moles^{-2} \cdot sec^{-1}$. The Arrhenius activation energy for the peroxidation of ferrocytochrome *c* was 9 kcal·mole^{-1} in the presence of the copper complex. The requirement for H_2O_2 suggests participation of a ternary complex and the following mechanism is the simplest consistent with this fact, the kinetics, and the stoichiometry of the process:

$$Cu^{2+}His + H_2O_2 \leftrightharpoons H_2O_2\text{–}Cu^{2+}His$$

$$\text{cyt. } c^{2+} + H_2O_2\text{–}Cu^{2+}His \longrightarrow \text{cyt. } c^{3+} + (HO)_2Cu^{+}His$$

$$\text{cyt. } c^{2+} + (HO)_2Cu^{+}His \longrightarrow \text{cyt. } c^{3+} + Cu^{2+}His + 2HO^{-}$$

After the cytochrome *c* had become completely oxidized, it slowly began to become reduced again until an equilibrium was reached with the cytochrome *c* about 60% to 70% reduced. This reduction could be duplicated by mixing ferricytochrome *c*, H_2O_2, and the copper complex, suggesting that cytochrome *c* was reduced in the disproportion of H_2O_2 by the copper complex.

INTRODUCTION

The oxidation of ferrocytochrome *c* by H_2O_2 is catalysed in cells by cytochrome *c* peroxidase, an enzyme which has been intensively studied by several workers[1–3]. We have previously shown that the oxidation of ferrocytochrome *c* by H_2O_2 in the absence of any added catalyst is product catalysed[4] and these findings have been confirmed

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recently by MOCHAN AND DEGN[5]. In other words, ferricytochrome *c* acts as a peroxidase, not merely toward organic reducing agents as reported by FLATMARK[6], but also toward ferrocytochrome *c*. We have previously shown also that when the oxidation of ferrocytochrome *c* in the presence of molecular oxygen was accelerated by a copper citrate complex[7] or by a copper histidine complex[8], significant amounts of H_2O_2 were not found among the products. It was possible that the ferricytochrome *c* in the products catalysed the reduction of any peroxide formed, to water. However, copper complexes also have been shown to catalyse the reduction of H_2O_2 by several classes of compound including *p*-phenylenediamine[9], guaiacol[10], and *p*-toluidine[11], and it was thus possible that the copper complex in the ferrocytochrome *c* oxygen system was acting as a cytochrome *c* peroxidase also.

Copper (bis) histidine was therefore tested for its ability to accelerate the oxidation of ferrocytochrome *c* by hydrogen peroxide. The copper complex studied was found to catalyse not only the oxidation but also the reduction of cytochrome *c* in the presence of H_2O_2. Reduction of cytochrome *c* in the presence of an active peroxidase system has been previously reported by YAMAZAKI[12], but in his system indole acetic acid was presumed to have acted as the reducing agent. In the experiments reported here, cytochrome *c* was reduced in the absence of any electron donor other than the H_2O_2 itself. It has been shown by BALLOU and coworkers[13] that cytochrome *c* may be reduced by superoxide ions. It has been postulated that superoxide ions may be involved in the catalase-like action of a copper histamine complex[14], and the present studies provide supporting evidence.

MATERIALS AND METHODS

Cytochrome *c* (Grade 1 Seravac Laboratories, Maidenhead, England) of 93% purity was reduced using H_2/Pd as described by SMITH[15].

Copper (bis) histidine was prepared in solution by dissolving 50 mg of hydrated copper sulphate and 76 mg of histidine monohydrochloride (*i.e.* a 1:2 molar ratio) in 10 ml of buffer pH 8.7. Addition of the histidine changed the colour to a deep blue and prevented precipitation of $Cu(OH)_2$ at higher pH values.

The buffer used throughout was prepared by mixing Kolthoff phosphate buffer stock solutions consisting of 0.1 M NaH_2PO_4 and of 0.05 M NaB_4O_7. A mixture of these solutions in the ratio 25:75, respectively, produced a pH of 8.7 and was used for all the reactions described here.

Unless otherwise specified, analytical grade reagents from British Drug Houses were used throughout, with deionized distilled water.

The reaction was followed using a Beckman DU2 spectrophotometer thermostatically regulated at 25°, with a Beckman 10″ recorder, to measure changes in the intensity of the 550 nm absorption band of ferrocytochrome *c*.

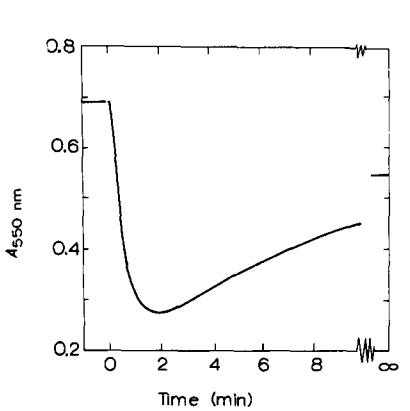
Buffer and cytochrome *c* were mixed in the cuvette to a final volume of 2.8 ml, then 0.1 ml of H_2O_2 was added. The mixture was observed for a few seconds to confirm that absorbance at 550 nm was constant, and the reaction was then initiated by addition of 0.1 ml of copper histidine solution on a polyethylene mixing plunger. Final concentrations are given in the legends to the figures. It was confirmed that changing the order of addition of the H_2O_2 and the copper histidine solutions did not change the experimental results. The pH was checked after the reactions to confirm that

no change had occurred. Fully oxidized and fully reduced readings were taken at the end of each reaction after addition of small excesses of potassium ferricyanide and sodium hydrosulfite.

Order of the reaction and the rate constant were determined by linear regression through appropriate functions of absorbance and time[16]. In determining the concentrations of cytochrome *c*, the difference in molar absorptivity at 550 nm between ferro- and ferricytochrome *c* was assumed to be $1.86 \cdot 10^4\ M^{-1} \cdot cm^{-2}$ (see ref. 17).

RESULTS

A pH of 8.7 was selected for the study of the effect of the copper complex, since at this pH interference by autoxidation and product catalysed peroxidation were found to be negligible. Addition of H_2O_2 to the ferrocytochrome *c* produced no detectible effect, but subsequent addition of the copper complex produced an immediate and marked enhancement in the rate of oxidation which proceeded until the cytochrome *c* was completely oxidised, as indicated by complete disappearance of the 550 nm peak. Likewise, addition of copper histidine alone caused no significant oxidation until the hydrogen peroxide was added. At lower pH however, the aerobic oxidation of ferrocytochrome *c* is greatly accelerated by the copper histidine complex[8]. Very soon after complete oxidation was attained, rereduction of the cytochrome *c* commenced which ceased when the cytochrome *c* was once again 60–70 % reduced. Fig. 1 shows the progress of a typical reaction. Repetitive scanning over the wavelength range 560–510 nm at the crucial stages of the reaction showed that during the course of the oxidation and rereduction both the 550 nm and 520 nm peaks of the cytochrome *c* completely disappeared and then reappeared in a form indistinguishable from those of native ferrocytochrome *c*.



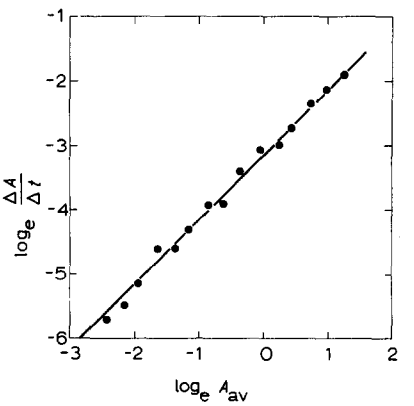


Fig. 1. Oxidation and rereduction of cytochrome *c* by H_2O_2 in the presence of copper histidine. H_2O_2 was added at zero time. The contents of the cuvette after this addition was: 22.5 μM with respect to cytochrome *c*, 330 μM with respect to copper, 292 μM with respect to H_2O_2. The reaction medium was 2 ml of Kolthoff phosphate buffer pH 8.7 diluted to a final volume of 3 ml by the other reagents. The temperature was 25°. Absorbance at 550 nm is plotted as a function of time.

Fig. 2. Order with respect to time, of the oxidation of ferrocytochrome *c* by H_2O_2 in the presence of copper histidine. Logarithm of the rate of change of absorbance per sec is plotted as a function of the logarithm of the difference between the average absorbance during the time interval chosen and the fully oxidizing reading. A straight line of slope 1 is plotted through the points, and the fit indicates a pseudo first order reaction.

The oxidative phase of the reaction was first order with respect to time (Fig. 2) since the only reactant changing in concentration during this period is cytochrome *c*, H_2O_2 being present in excess. Varying the initial concentrations of H_2O_2 and of the copper complex produced proportionate changes in the pseudo first order rate constant (Figs. 3 and 4), while changing the initial concentration of ferrocytochrome *c* changed

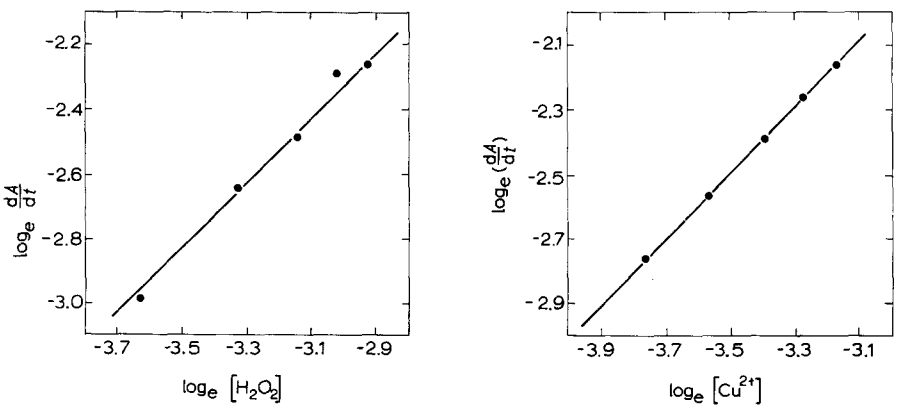


Fig. 3. Dependence of initial rate of oxidation of ferrocytochrome *c* on the initial concentration of H_2O_2. Conditions were as stated in Fig. 1 except that initial H_2O_2 concentration was as indicated on the graph. Logarithm of the initial rate of absorbance change is plotted as a function of the initial molar concentration of H_2O_2. A straight line of slope 1 has been drawn through the points.

Fig. 4. Dependence of initial rate of oxidation of ferrocytochrome *c* on the concentration of copper histidine complex. Conditions were as stated in Fig. 1 except that copper concentration was as indicated on the graph. Logarithm of the initial rate of absorbance change is plotted as a function of the logarithm of the total molar concentration of copper. A straight line of slope 1 has been drawn through the points.

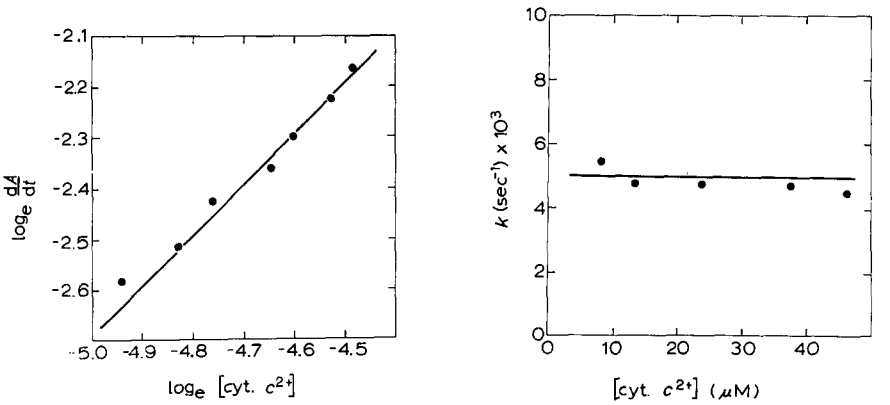


Fig. 5. Dependence of initial rate of oxidation of ferrocytochrome *c* on initial concentration of ferrocytochrome *c*. Conditions were as stated in Fig. 1 except that the initial ferrocytochrome *c* concentration was as indicated on the graph. Logarithm of the initial rate of absorbance change (sec^{-1}) is plotted as a function of the logarithm of the initial molar concentration of ferrocytochrome *c*. A straight line of slope 1 has been drawn through the points.

Fig. 6. Effect of initial concentration of ferrocytochrome *c* on the pseudo first order rate constant for the oxidation of ferrocytochrome *c*. Conditions were as stated in Fig. 5. No systematic effect is evident.

the initial velocity proportionately (Fig. 5) without producing any significant change in the rate constant over the range of concentrations which could be accurately followed (Fig. 6).

The reductive phase of the reaction could be initiated directly by starting with ferricytochrome *c* rather than ferrocytochrome *c* in the cuvette. Neither H_2O_2 alone nor the copper histidine alone caused reduction, but the presence of both duplicated the effect observed at the end of the oxidative phase.

The effect of temperature on the copper catalysed reaction was studied and a linear plot of the logarithm of the pseudo first order rate constant against the reciprocal of the absolute temperature was obtained (Fig. 7), from which Arrhenius activation energy of 9.0 kcal·mole^{-1} was calculated.

A study of the effect of a range of concentrations of copper on the absorption of ferricytochrome *c* at 690 nm was undertaken. No effects were observed under the conditions described, even at 1 mM copper histidine complex and 150 μM cytochrome *c*.

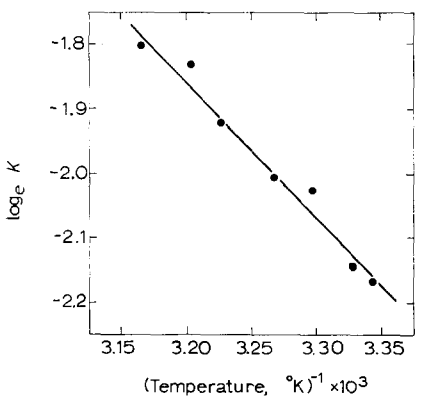


Fig. 7. The effect of temperature on the rate of oxidation of ferrocytochrome *c*. Conditions were as stated in Fig. 1 except that the temperature was as indicated in the graph. Logarithm of the pseudo first order rate constant is plotted against 10^3 times the reciprocal of the absolute temperature. A slope of $-2.0 \cdot 10^{-3}$ was obtained for the best straight line through these points. This corresponds to an Arrhenius activation energy of 8.7 kcal·mole^{-1}.

DISCUSSION

The kinetic behaviour of the oxidative phase is that of a simple third order reaction, the process exhibiting first order dependence on each of the three reactants. The pseudo first order behaviour results from the fact that only the ferrocytochrome *c* concentration changes appreciably during this part of the reaction, the other reagents being present in excess. The third order rate constant under our experimental conditions was $5 \cdot 10^4$ l^2·mole^{-2}·sec^{-1}.

In view of the catalysis by copper of the above mentioned peroxidations, this catalysis of the peroxidation of cytochrome *c* is perhaps not surprising. The rate dependence of the reaction is likewise consistent with several simple mechanisms involving participation of copper, H_2O_2, and ferrocytochrome *c* prior to the rate determining step. The third order character of the reaction, presumably results from

a sequence of bimolecular steps, since ternary collisions are rare. The reaction is thus a multistep process, but the nature and sequence of the steps cannot be distinguished with certainty from the present kinetic data. Certain conclusions can be drawn, however. The fact that the copper histidine complex catalyses peroxidation of ferrocytochrome *c* but not its oxidation by molecular oxygen under these conditions implies that reaction of copper with H_2O_2 precedes oxidation of the ferrocytochrome *c*, for if the initial step was the direct oxidation of ferrocytochrome *c* by copper, the molar excess of copper should allow the reaction to proceed at a significant rate in the absence of H_2O_2 and even in the absence of oxygen. It seems clear therefore that under the conditions chosen, electron transfer occurs only in a ternary complex. The stoichiometry of the process demands further that two successive reactions with cytochrome *c* occur to complete the reduction of H_2O_2 to water. The following scheme is the simplest mechanism consistent with the kinetics, the lack of reactivity toward oxygen, and the stoichiometry:

$$Cu^{2+}His + H_2O_2 \leftrightharpoons H_2O_2\text{–}Cu^{2+}His \quad (1)$$

$$\text{cyt. } c^{2+} + H_2O_2\text{–}Cu^{2+}His \longrightarrow \text{cyt. } c^{3+} + (HO)_2Cu^{+}His \quad (2)$$

$$\text{cyt. } c^{2} + (HO)_2Cu^{+}His \longrightarrow \text{cyt. } c^{3+} + Cu^{2+}His + 2HO^{-} \quad (3)$$

The species shown represent the sequence of events and are not intended to denote the structure of the intermediates. Alternative schemes in which cytochrome *c* reacts initially with the copper complex, or with hydrogen peroxide are equally consistent with the kinetics, but less likely in view of the fact that no change is observed when ferrocytochrome *c* is incubated with either of these in the absence of the other.

The first order dependence of initial rate on substrate concentration indicates that Step (3) is fast relative to the other two steps otherwise the reaction would be second order with respect to ferrocytochrome *c*. The first order dependence with respect to time shows also that Steps (2) and (3) are relatively irreversible, for otherwise the accumulation of products would contribute to slowing the reaction.

The acceleration by copper of the reduction of ferricytochrome *c* by H_2O_2 was unexpected in view of the fact that a transient equilibrium or steady-state was observed, with the cytochrome *c* fully oxidized. Evidently after oxidation of the cytochrome *c* is completed, some change in the composition of the reaction medium displaces the equilibrium or steady-state.

Superoxide anions are known to reduce ferricytochrome *c* (ref. 13), and disproportionation of the H_2O_2 by the copper complex[14] is thought to be mediated by superoxide ions. These are therefore the most likely candidates for the role as reducing agent. The data show that a steady state in which the cytochrome *c* is in the oxidized form shifts to a situation in which most of the cytochrome *c* is in the reduced form. Due to the presence of copper in our system, disproportionation of the H_2O_2 presumably continued even after the ferrocytochrome *c* had been oxidized. When the peroxide concentration had decreased sufficiently, reduction of the cytochrome *c* by superoxide should become faster than its reoxidation, with the observed results. Possible modification of the cytochrome *c* by copper ions was considered, but this was not evident in the spectrum produced by excess sodium dithionite or upon oxidation with potassium ferricyanide. In particular, the absence of an effect of copper histidine

on the 690 nm peak of ferricytochrome *c* seems to exclude the possibility of significant conformational changes involving the haem crevice, caused by the copper.

It is concluded that these results provide experimental evidence supporting the involvement of superoxide ions in the catalyse-like action of copper complexes.

The demonstration that a soluble, relatively simple, compound carries out the same reaction as the enzyme cytochrome *c* peroxidase raises the question of possible analogies in the mechanism. The activation energy of 9.0 kcal·mole^{-1} can perhaps be compared with values of 16.0 kcal·mole and 11.5 kcal·mole^{-1} for the non-enzymic reaction at pH 8.1 and 6.6 (using a higher concentration of H_2O_2), and with the value of 9.3 kcal·mole^{-1} for the enzymic reaction, calculated from the data of BEETLESTONE[2]. While the catalyst, like the enzyme[2,18] lowers the activation energy for the peroxidation of ferrocytochrome *c*, such analogies are significant mainly in emphasizing the need for further studies of both systems, to enable comparisons to be made with more confidence.

ACKNOWLEDGEMENT

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