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Some New Details of the Copper-Hydrogen Peroxide Interaction

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The addition of neocuproine (NC) or bathocuproinedisulphonate at the end of the autooxidation of Cu^{I} in phosphate buffer, pH 7.4, regenerates almost entirely the O_2 consumed. Other chelating agents assayed, including *o*-phenanthroline, cannot replace NC in promoting the O_2 formation. O_2 is also produced by adding NC to a mixture of Cu^{II} and H_2O_2. Concomitant with the O_2 evolution, the typical absorbance of the $(NC)_2Cu^{I}$ complex appears to account for the complete reduction of Cu^{II} to Cu^{I}. It is concluded that the addition of H_2O_2 with Cu^{II} produces the equilibrium $Cu^{II}(O_2H)^{-} \leftrightarrow Cu^{I}{}^{\bullet}O_2H$. Addition of NC shifts the equilibrium to the right side by binding Cu^{I}. The released $O_2^{\bullet -}$ then reacts with the remaining Cu^{II} yielding, in the presence of NC, the net reaction of 4 NC + 2 Cu^{II} + H_2O_2 → 2 $(NC)_2Cu^{I}$ + O_2 + 2 H^{+}. O_2 is also released in the absence of added NC provided the H_2O_2 concentration is increased. In these conditions the $Cu^{II}(O_2H)^{-}$ complex undergoes other reactions leading to the copper-catalysed decomposition of H_2O_2.

Interaction of copper with H_2O_2 has been investigated in the past as a producer of oxygen reactive species known to induce extensive biological damages, comparable to those produced by the more popular iron Fenton reaction (1-5). Though most of the aspects of the Fenton and of the Cu-Fenton-like reactions are understood, some details remain to be elucitated. In a recent study on the copper catalysed oxidation of cysteine (6) we found that the presence of H_2O_2, in mixtures where redox changes of copper were followed by the neocuproine (NC) assay, could induce a fast reducton of Cu^{II} to Cu^{I} except when the assay was performed at pH 4-4.5. On the basis of these findings we thought of interest to investigate further the mechanism of the H_2O_2-Cu^{II} interaction in the presence and absence of neocuproine (NC). The preliminary results of this study are reported in this note.

Abbreviations: NC, neocuproine-HCl; OP, *o*-phenanthroline; EDTA, ethylenediaminetetracetic acid; DTPA, diethylenetriaminepentacetic acid; MOPS, 3-morpholinopropanesulfonic acid.

MATERIALS AND METHODS

Catalase was purchased from Sigma Co. Chemicals were of the highest quality from Sigma, Fluka and Merck. Cuprous chloride was dissolved in N_2-saturated CH_3CN immediately before use. Quantitative determination of the Cu^{I}-neocuproine chelate was performed at 454 nm using ϵ = 7,95O M^{-1} cm^{-1} (7). H_2O_2 concentration was measured at 23O nm using a molar extinction coefficient of 72.4 (8). Spectrophotometric analyses were carried out with UVICON 94O (Kontron) equipped with a thermostated cell compartment and with a magnetic stirrer. O_2 in solution was measured polarographically (Gilson Oxygraph) using a 1.8 ml reaction chamber at 25°C unless stated otherwise.

RESULTS

Fig 1A illustrates the autoxidation of 12O μM Cu Cl in phosphate buffer, pH 7.4 in air-equilibrated solution. The oxidation stops when the amount of O_2 is consumed accounting for the oxidation of Cu^{I} to Cu^{II}, with production of H_2O_2. The unexpected finding is that about all the O_2 consumed is regenerated on adding 1 mM NC. The reaction is the same by substituting phosphate with the buffer MOPS at the same pH 7.4. Bathocuproinedisulphonate exhibits the same effect, but not *o*-phenanthroline (OP), EDTA, DTPA, thiourea, histidine and ethylenediamine. Lowering the pH to 4 halts the regeneration of O_2 on adding NC (not shown). When the Cu^{I} autoxidation is done in the presence of O.1 mg/ml catalase no O_2 production is observed after the addition of NC. Autoxidation of Cu^{I} is known to proceed as in reactions 1-3)

$$1)\quad Cu^{I} + O_2 \rightarrow Cu^{II} + O_2^{\bullet -}$$

$$2)\quad Cu^{I} + O_2^{\bullet -} + 2\,H^{+} \rightarrow Cu^{II} + H_2O_2$$

$$\text{Sum 3)}\quad 2\,Cu^{I} + O_2 + 2\,H^{+} \rightarrow 2\,Cu^{II} + H_2O_2$$

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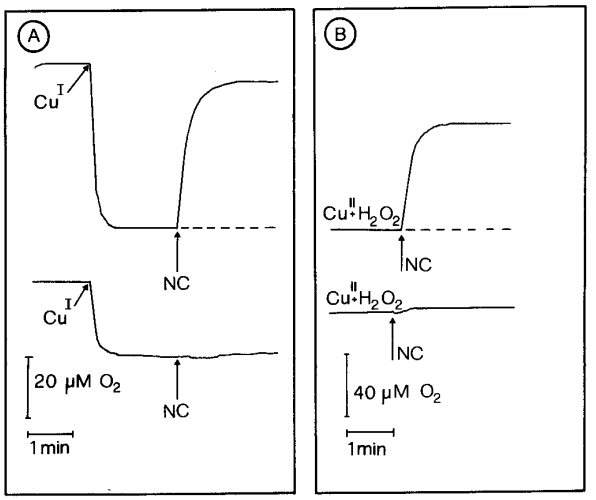

FIG. 1. (A) Polarographic measurements of oxygen in solution at 32°C in 5O mM K phosphate buffer, pH 7.4, following the addition of 12O μM CuCl with subsequent addition of 1 mM NC at the arrow (upper trace). The same as above but with O.1 mg catalase/ml added at zero time (lower trace). (B) 2OO μM $CuCl_2$ plus 1OO μM H_2O_2 with subsequent addition of 1 mM NC, at the arrow, in the absence (upper trace) and in the presence (lower trace) of 4OO μM EDTA. The broken line is obtained by substituting NC with OP, EDTA, and other complexants (see text).

with also the transient production of $^{\bullet}OH$ from H_2O_2-Cu^{I} interaction (9-11). Because at end of oxidation the final products are only 2 Cu^{II} and H_2O_2, the addition of NC has been assumed to have shifted the reaction 3) to the left by binding Cu^{I} (see Eq. 8, below). On the basis of this conclusion, the addition of NC to H_2O_2 and Cu^{II} in phosphate buffer, pH 7.4, should display the same effect as that observed by adding NC at end of Cu^{I} autoxidation. This is the case as seen in Fig. 1 B. Again the other chelating agents assayed (OP, EDTA, DTPA, thiourea, histidine and ethylendiamine) cannot replace NC in promoting the O_2 formation. Yet, evolution of O_2 is prevented by EDTA (Fig. 1 B, lower trace) or DTPA (not shown) added before the NC in the ratio 2/1 with respect to Cu^{II} . By relating O_2 production with H_2O_2 concentration, it is found that the stoichiometry of the reaction is 1 mol of O_2 per mol of H_2O_2 reacted with 2 Cu^{II} (Fig. 2). Reaction 8) can be followed also by the formation of the colored $(NC)_2Cu^{I}$ chelate. Fig. 3 A shows that the absorbance of Cu^{II} and of NC are not appreciably changed by adding H_2O_2, while the typical absorbance (λ max = 454 nm) of the cuprous-NC chelate is produced when the three reactants are added together. The molar ratio of $(NC)_2Cu^{I}$, determined spectrophotometrically, and the O_2 produced (Oxygraph), under the same conditions, has been found to be 2.1 in good agreement with the stoichiometry of reaction 8). The pH dependence of this reaction is shown in Fig. 3 B.

We studied also the H_2O_2-Cu^{II} interaction in the absence of NC. Fig. 4 A and B show that the production of O_2 occurs in the absence of NC provided the concentration of H_2O_2 is higher than that used in the presence of NC. The O_2 production is strictly related to H_2O_2 concentration and is pH dependent. EDTA or DTPA added in twofold excess to the Cu^{II} concentration completely prevent the O_2 evolution (not shown). OP cannot replace NC in the Cu^{II}-H_2O_2 system. Since OP is a strong chelator of Fe^{II}, like NC is for Cu^{I}, we tried to substitute Cu^{II} with Fe^{III} and NC with OP, to see whether Fe^{III} could display the effect similar to that shown with Cu^{II}. No color of the Fe^{II} OP complex was seen, neither O_2 was produced in the Oxygraph. The reaction, thus, appears specific for the Cu^{II}-H_2O_2-NC system. No other transition metals, however, have been assayed.

DISCUSSION

The data reported in this note indicate that Cu^{II} and H_2O_2 interact producing free O_2 in two distinct ways, one by adding the Cu^{I} specific chelator NC (or bathocuproinedisulphonate) the other by adding an excess of H_2O_2 in the absence of any chelator. The O_2 determined in the Oxygraph has been in any case a valuable tool to appreciate the occurrence of these two reactions. As a possible mechanism of interaction we suggest that H_2O_2 binds in a coordination site of Cu^{II} in the place of a water molecule, or of other ligands of affinity lower than H_2O_2 (i.e. buffer) as shown in reaction 4)

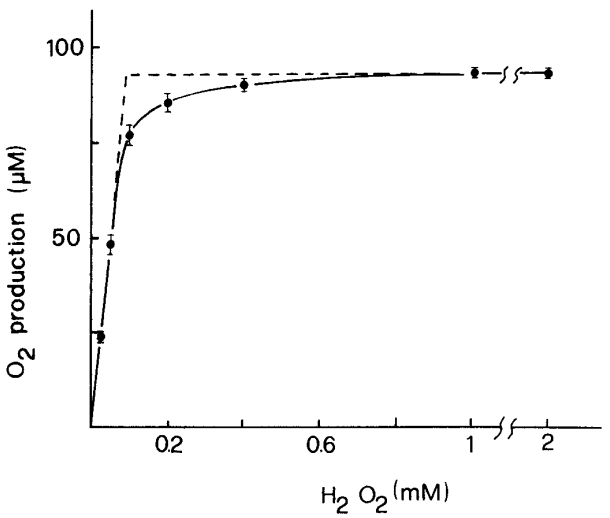

FIG. 2. Oxygen release as a function of H_2O_2 concentration. 2OO μM $CuCl_2$ was added with H_2O_2 (O.O25 to 2 mM) in 5O mM phosphate buffer, pH 7.4 , at 25°C. The O_2 produced on the addition of 1 mM NC was determined at the end of reaction by the Oxygraph.

4) $Cu^{II} + H_2O_2 \rightarrow Cu^{II}(O_2H)^- + H^+$

5) $Cu^{II}(O_2H)^- \leftrightarrow Cu^{I}{}^{\bullet}O_2H$

6) $Cu^{I}{}^{\bullet}O_2H + 2\,NC \rightarrow (NC)_2Cu^{I} + O_2^{\bullet -} + H^+$

7) $2\,NC + Cu^{II} + O_2^{\bullet -} \rightarrow (NC)_2Cu^{I} + O_2$

Sum 8) $4\,NC + 2\,Cu^{II} + H_2O_2 \rightarrow 2\,(NC)_2Cu^{I} + O_2 + 2\,H^+$

As shown in 5) the $Cu^{II}(O_2H)^-$ complex is assumed to be in equilibrium with the Cu^{I}-superoxide complex by the reversible exchange of an electron. The occurrence of such transient complexes have been proposed and discussed in the past (12, 13). On the addition of NC the equilibrium 5) is pushed to the right side with formation of the stable $(NC)_2Cu^{I}$ chelate and generation of the superoxide radical (react. 6). This radical then reduce another Cu^{II} to Cu^{I} (14) releasing free O_2

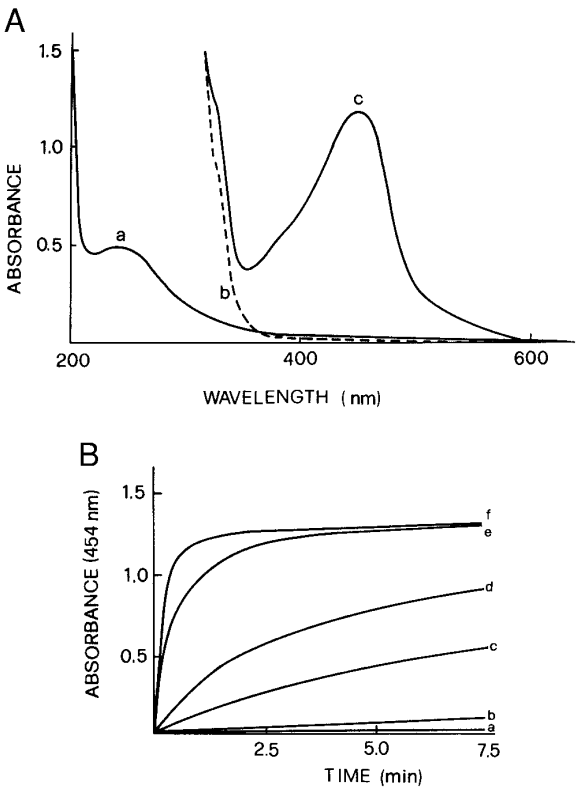

FIG. 3. (A) absorbance of (a) 2OO μM $CuCl_2$ in the presence and in the absence of 1OO μM H_2O_2; (b) 1 mM NC in the presence and in the absence of 1OO μM H_2O_2; (c) 2OO μM $CuCl_2$ plus 1OO μM H_2O_2 added with 1 mM NC. (B) Time course of copper reduction by H_2O_2, in the presence of NC (absorbance at 454 nm) at different pHs. The reacting mixture contained 2OO μM $CuCl_2$, 1OO μM H_2O_2 and 1 mM NC in 5O mM phosphate buffer at pH (a) 4.5, (b) 5.25, (c) 6.25, (d) 6.6, (e) 7.4, and (f) 8. Copper addition to start the reaction. In separate assays we have verified that the rate of formation and the 454 nm extinction of the cuprous-NC complex (CuCl added with fivefold excess of NC) are the same in the pH range 4–8.

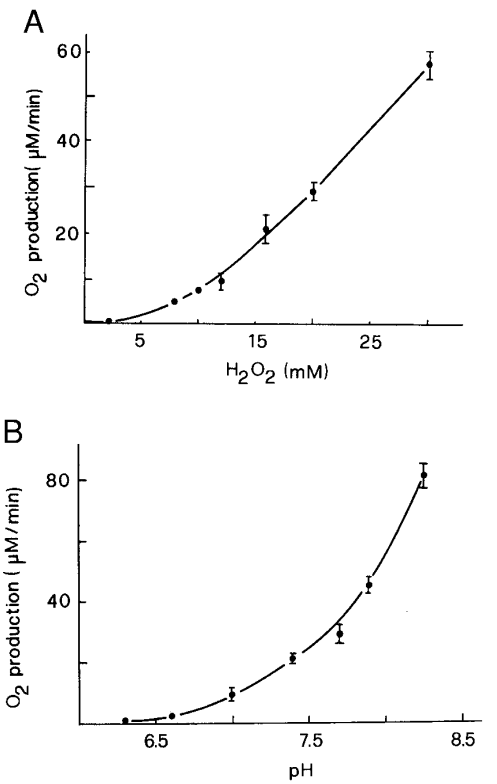

FIG. 4. (A) Rate of oxygen production on the addition of 2OO μM $CuCl_2$ with different H_2O_2 concentrations (2–2O mM) in 5O mM phosphate buffer, pH 7.4. (B) 2OO μM $CuCl_2$ with 16 mM H_2O_2 in 5O mM phosphate buffer at different pHs. 25°C.

(react. 7). The overall reaction occurring in the presence of NC is represented in Eq. 8. Similarly to reactions 5) and 6), it has been reported that interaction of superoxide dismutase by H_2O_2 induces reduction of the copper of the enzyme and generation of $O_2^{\bullet -}$ (15).

A likely explanation of the O_2 production in the absence of NC with excess of H_2O_2 is presented in reactions 9-12).

9) $Cu^{II} + H_2O_2 \rightarrow Cu^{I}{}^{\bullet}O_2H + H^+$

10) $Cu^{I}{}^{\bullet}O_2H + H_2O_2 \rightarrow Cu^{I} + O_2 + {}^{\bullet}OH + H_2O$

11) $Cu^{I} + H_2O_2 \rightarrow Cu^{II} + {}^{\bullet}OH + OH^-\ Cu^{II}$

Sum 12) $3H_2O_2 \rightarrow O_2 + 2\,{}^{\bullet}OH + 2\,H_2O$

Reaction 1O) is similar to the Haber-Weiss mechanism while reaction 11) is the copper-driven Fenton reaction. Altogether these reactions produce O_2 and a relevant amount of $^{\bullet}OH$ (reaction 12) with copper acting catalytically. The radical $^{\bullet}OH$ is expected to react with the excess of H_2O_2 (16) or dimerize (17) to regenerate part of H_2O_2 consumed. The notable production of hydroxyl radical could account for the reported higher deleteri-

ous effects of the $Cu^{II} + H_2O_2$ system to biological structures compared with the corresponding $Fe^{III} + H_2O_2$ system (3, 5, 18).

The activating effect of alkalinity, either in the presence and in the absence of NC (Fig. 3 B and 4 B), suggests the deprotonation of the Cu^{II}-H_2O_2 complex as a favourable condition.

A plausible alternative to the mechanism reported above could be the possible binding of H_2O_2 with two Cu^{II} ions, yelding the hydroperoxo-dicopper complex (19) and the formation of O_2 by two-electron transfer, without the intermediate production of $O_2^{\bullet -}$.

An important outcome of the reported data is also that the determination of the ratio Cu^{I}/Cu^{II} cannot be done with NC (or with bathocuproinedisulphonate) in the presence of H_2O_2 unless the analysis is done at pH below 5 or the peroxide is previously removed.

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