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# THE ROLE OF COPPER IN THE CATALYTIC ACTION OF LACCASE AND CERULOPLASMIN

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## SUMMARY

1. The rate of reoxidation of ascorbic acid-reduced copper in laccase (EC 1.10.3.2) and ceruloplasmin has been measured spectrophotometrically in a stopped-flow apparatus. The kinetics of reduction of $Cu^{2+}$ by various substrates has been studied by electron-spin resonance absorption. The rate of formation and decay of a free radical formed with *p*-phenylenediamine as substrate has also been measured, and the radical has been identified as the positive ion.

2. The rate of $O_2$ consumption, calculated from the kinetic parameters on the basis of a mechanism involving reduction of $Cu^{2+}$ by the substrate followed by reoxidation by $O_2$, agrees well with the experimentally determined rate, thus providing strong evidence for the correctness of this mechanism.

3. In a few cases, the experimental curves for $Cu^{2+}$ and free radical have been compared with calculated curves, obtained by numerical integrations of kinetic equations with a digital computer. The results indicate that the radical decay occurs by a reaction not involving the enzymes.

4. Electron-spin resonance measurements of a bacterial copper protein, azurin, indicate that small hyperfine splittings, as observed with laccase and ceruloplasmin, may occur without an exchange interaction between $Cu^{2+}$ and $Cu^{1+}$. It is suggested that the $Cu^{1+}$ of laccase and ceruloplasmin may bind the substrates by interacting with their $\pi$-electrons.

## INTRODUCTION

In a previous study[1] it was shown by the ESR technique that laccase (*p*-diphenol:$O_2$ oxidoreductase, EC 1.10.3.2) contains $Cu^{2+}$ which becomes partially reduced on addition of substrate. While this lends support to the common assumption (see, for example, ref. 2) that the mechanism of action of this enzyme involves a reduction of the metal by the substrate, followed by reoxidation by $O_2$, a clear de-

Abbreviations: ESR, electron-spin resonance; PPD, *p*-phenylenediamine; DPP, *N,N'*-dimethyl-*p*-phenylenediamine.

monstration of this role of the metal in catalysis requires a correlation of the kinetics of copper reduction and oxidation with the rate of the overall reaction. We have consequently studied the rate of change in copper valency with different substrates by measurements of ESR and visible absorption with laccase, as well as with ceruloplasmin, the copper-protein of mammalian serum. Earlier ESR studies[3,4] have shown that these two proteins contain $Cu^{2+}$ bonded in almost identical manner, and they also show similar enzymic specificity, but the specific activity of ceruloplasmin is considerably lower than that of typical copper oxidases[5], such as laccase (BROMAN, unpublished). Thus, a more detailed study of the kinetics by the ESR method has only been possible with ceruloplasmin.

Recently it has been shown[4,6] that both laccase and ceruloplasmin, in addition to $Cu^{2+}$, contain an approximately equimolar amount of $Cu^{1+}$. It has been suggested[7] that an exchange interaction between $Cu^{2+}$ and $Cu^{1+}$ is related to the characteristic ESR spectra of these two proteins. ESR measurements on the bacterial copper protein, azurin, which contains only 1 copper atom per molecule[8], indicate that this is not a unique interpretation of the spectral properties, and a possible function of $Cu^{1+}$ will be discussed.

## MATERIALS AND METHODS

### *Proteins*

Fungal laccase *A* was prepared by the method of MOSBACH[9]. The concentration of total copper in laccase solutions was calculated from the absorbancy at 610 mμ by use of the known extinction coefficient[9]. Ceruloplasmin was obtained from AB Kabi, Stockholm (we are indebted to Mr. H. BJÖRLING for generous gifts of this material). The preparation used was about 80% pure, as determined from the ratio of absorbancies at 610 and 280 mμ[10]. The content of specifically bound copper in solutions of the protein was calculated from the absorbancy at 610 mμ[10].

For all kinetic experiments the protein solutions were dialyzed against the buffer used (usually 0.1 M triethylamine–acetic acid, pH 5.5).

Azurin, prepared according to SUTHERLAND AND WILKINSON[8], was kindly supplied by Dr. I. W. SUTHERLAND. Its purity was checked by zone electrophoresis in phosphate–borate buffer (*I* 0.05, pH 8.2) and ESR spectra were recorded with frozen solutions in the same buffer or in water. The ratio of the absorbancy at 625 and 280 mμ was 0.47 for the sample used (*cf.* ref. 8).

### *Chemicals*

PPP and triethylamine were purchased from Eastman Organic Chemicals; PPD from Riedel-de Haën; catechol from Merck, Darmstadt; quinol from May and Baker; pyrogallic acid from Mallinckrodt; and the disodium salt of EDTA from Hopkin and Williams.

### *Determination of the rate of reoxidation of reduced laccase and ceruloplasmin*

Solutions of laccase and ceruloplasmin containing about $6 \cdot 10^{-4}$ M $Cu^{2+}$ (*cf.* refs. 4, 6) were used. A volume (7 ml) just sufficient to cover the bottom was introduced into flask D of the apparatus shown in Fig. 1. The rubber stopper was tightly replaced and a short piece of plastic tubing at the upper end of the capillary E was clamped off. The entire system, including the football bladder C, was then evacuated with a water

pump and refilled with argon, which had been freed of traces of $O_2$ by bubbling through an alkaline pyrogallol solution. Evacuation and refilling were repeated several times. Finally the system was filled with argon at a slightly elevated pressure, so that C became well distended, and the apparatus closed off by means of the stopcock, as shown in Fig. 1. A solution of 0.01 M ascorbic acid was prepared under argon in

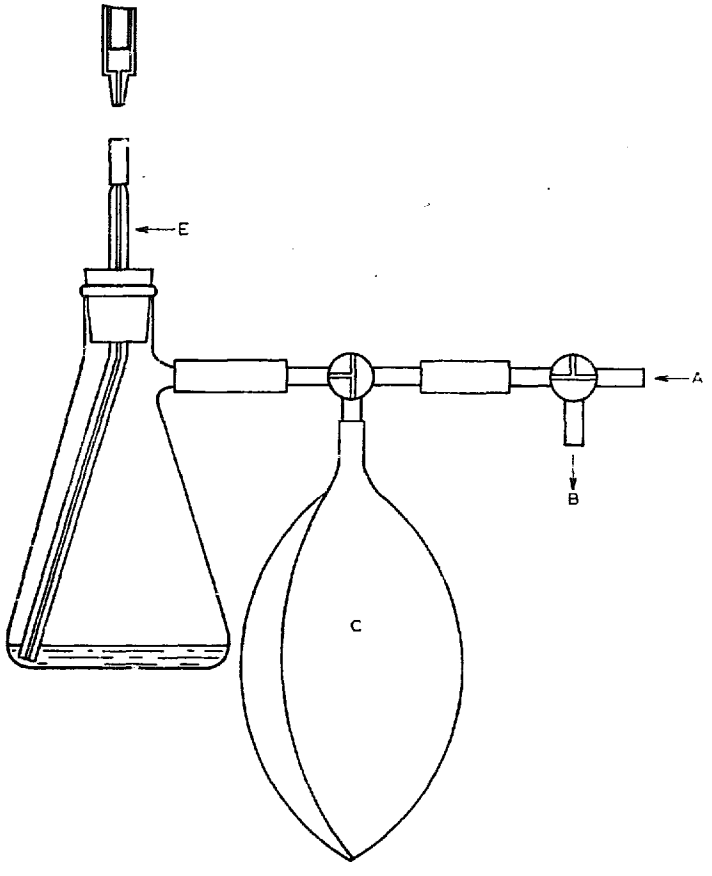


Fig. 1. Apparatus for anaerobic reduction of ceruloplasmin and laccase. The protein solutions were contained as thin layers in the vacuum flask D. C is a football bladder. During deaeration D is successively evacuated by a water pump, connected to B and filled with argon through A. Titration of the protein and removal of reduced samples is performed through the capillary E, the upper end of which has a short piece of tubing which can be clamped off.

a Thunberg tube and introduced into an Agla syringe under a stream of argon. Before the syringe, mounted in a micrometer holder, was connected to the tubing of the capillary E (Fig. 1), the flask was tilted to the right and the clamp removed, causing a stream of argon to pass from C through E, so as to prevent contamination with air. Two-thirds of the amount of ascorbic acid just sufficient to reduce all $Cu^{2+}$ (0.12 ml) was introduced and thoroughly mixed with the contents of flask D. D was then inclined as before, remaining ascorbic acid solution in E was sucked back into the syringe, and the clamp again applied. The solution was allowed to stand in a cold room (4°) overnight to allow complete reaction (*cf.* ref. 11). Samples were transferred anaerobically by connecting a Luer-Lok syringe, with the piston removed, to E and thoroughly flushing it with argon from C. The argon jet was shut off by cautiously replacing the piston. Flask D was then inclined to the left to allow filling of the syringe with the partially reduced protein solution. The solution was then mixed with aerated

buffer solution in a stopped-flow apparatus as described by SPENCER AND STURTEVANT[12], and the rate of increase in absorption at 610 mμ was recorded. The apparatus as operated allowed the measurement of reactions with a half-life of about 0.05 sec.

*ESR experiments*

ESR absorption was recorded in a Varian V-4500 3-cm spectrometer using 100 kcycles/sec field modulation with the sample in a flat and thin (0.3 mm) quartz cell connected to the mixing chamber of a stopped-flow apparatus by a piece of thin plastic tubing. The time required for a solution to reach the cuvette after mixing was about 0.1 sec, as estimated from some experiments with radicals when the radical concentration was extrapolated back to zero. The cuvette was cooled by a stream of cooled $N_2$ gas through the cavity. The temperature of the cell was measured by means of a thermocouple in the cavity close to the cell. In a separate experiment, the temperature inside the cell was measured to check the effect of the microwave heating, and it was found that at the microwave power level used, the inside temperature was close to the temperature outside the cell. Two recorders were connected to the ESR instrument, one Varian G-10 recorder (response time 1 sec, paper speed 2 in/min) through a filter with a time constant of 0.3 sec, and a Brush RD 232100–561500 recorder (paper speed 0.5 cm/sec) connected so that the effective time constant was about 0.05 sec.

To follow the time course of a signal, the magnet field was set for the recording of maximum signal and the absorption recorded as a function of time. However, in the cases where a moderately strong radical signal appeared, the $Cu^{2+}$ decay was followed at a magnetic field slightly lower than that of maximum $Cu^{2+}$ signal to avoid mixing of the two signals. The modulation field was in most cases 41 gauss peak-to-peak for both $Cu^{2+}$ and radical signals. At least part of the latter signal was then overmodulated. This has the advantage that the effect of small changes during the reaction in the inherent line width of narrow radical signals could be neglected. Such overmodulation does not alter the integrated intensity[13]. In all cases the amplitude of the signal was taken as a measure of the total absorption. Some radical spectra were recorded at high resolution (modulation field 0.3 gauss).

## RESULTS

The rate of reoxidation of ascorbic acid-reduced laccase as well as ceruloplasmin was too rapid to allow measurement in the apparatus used, as more than 95% of the reduced copper* was reoxidized in 0.1 sec. The concentration of reduced copper in these experiments was about $2 \cdot 10^{-4}$ M and that of $O_2$ $1.5 \cdot 10^{-4}$ M. The buffer was here as in most other experiments 0.1 M triethylamine–acetic acid (pH 5.5).

Fig. 2 shows the initial part of the actual tracing of $Cu^{2+}$ ESR absorption as a function of time, obtained on the Varian recorder when ceruloplasmin was mixed with a catechol solution saturated with air. For accurate measurements of the early part of the curves, particularly with better substrates, the faster recorder had to be employed. The entire time course was reconstructed from measurements with both recorders, as illustrated with pyrogallol, catechol and PPD, in Figs. 3 and 4; Fig. 4

* It should be noted that this does not correspond to the total copper content, as about half the copper is present as $Cu^{1+}$ even in the oxidized form of the enzyme[4,6].

also includes radical ESR absorption (see below) and calculated curves (see DISCUSSION). Other substrates studied were quinol and hydroxylamine. The measured rates of reduction of $Cu^{2+}$ expressed as the time $T_{\frac{1}{2}}$ for half-reduction to the steady-state level, the duration of the steady state ($T_s$) and other kinetic parameters, estimated from such curves as described in the DISCUSSION, are summarized for the various substrates in Table I.

With laccase the rate of reduction was too fast to be measured, even on the fast recorder, with all substrates tested when a comparable concentration of protein

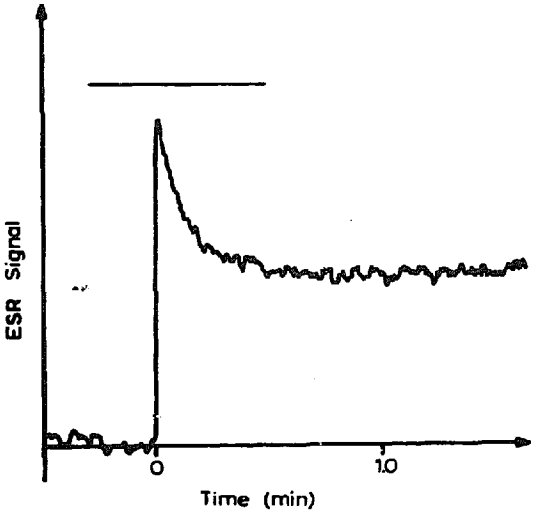


Fig. 2. Decay of ESR signal of $Cu^{2+}$ in an experiment with ceruloplasmin and catechol recorded with the low-response recorder. At time zero, the stopped-flow apparatus was activated. The horizontal line above the recorded curve indicates the signal level of ceruloplasmin with the same concentration of $Cu^{2+}$ in the absence of substrate. For concentrations, see Fig. 3. Here and in Figs. 3–5 the temperature of the cell was about 17°. The modulation field was 41 gauss (also in Figs. 3 and 4).

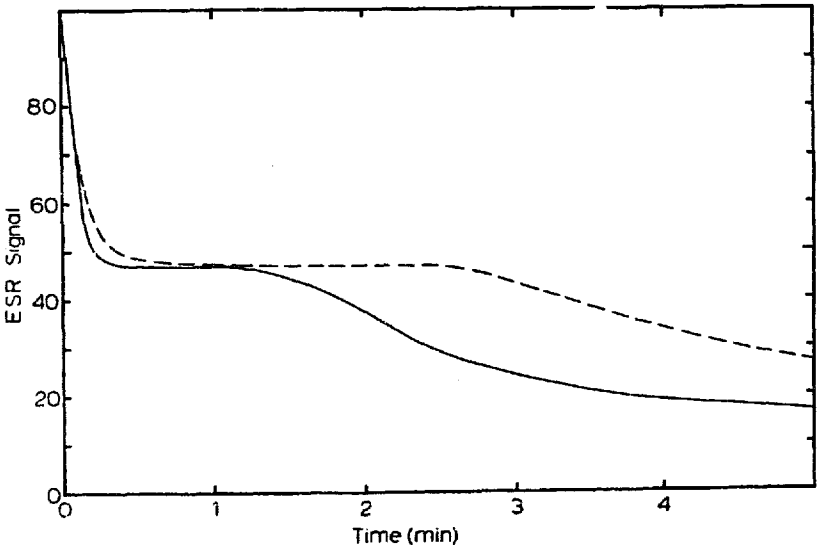


Fig. 3. Decay curves of ESR signal of $Cu^{2+}$ in ceruloplasmin obtained from recordings on the high- and low-response recorders. Concentrations immediately after mixing of total copper about $8 \cdot 10^{-4}$ M and of substrates (solid curve, pyrogallol; dashed curve, catechol) $5 \cdot 10^{-3}$ M. Buffer 0.1 M triethylamine–acetic acid (pH 5.5).

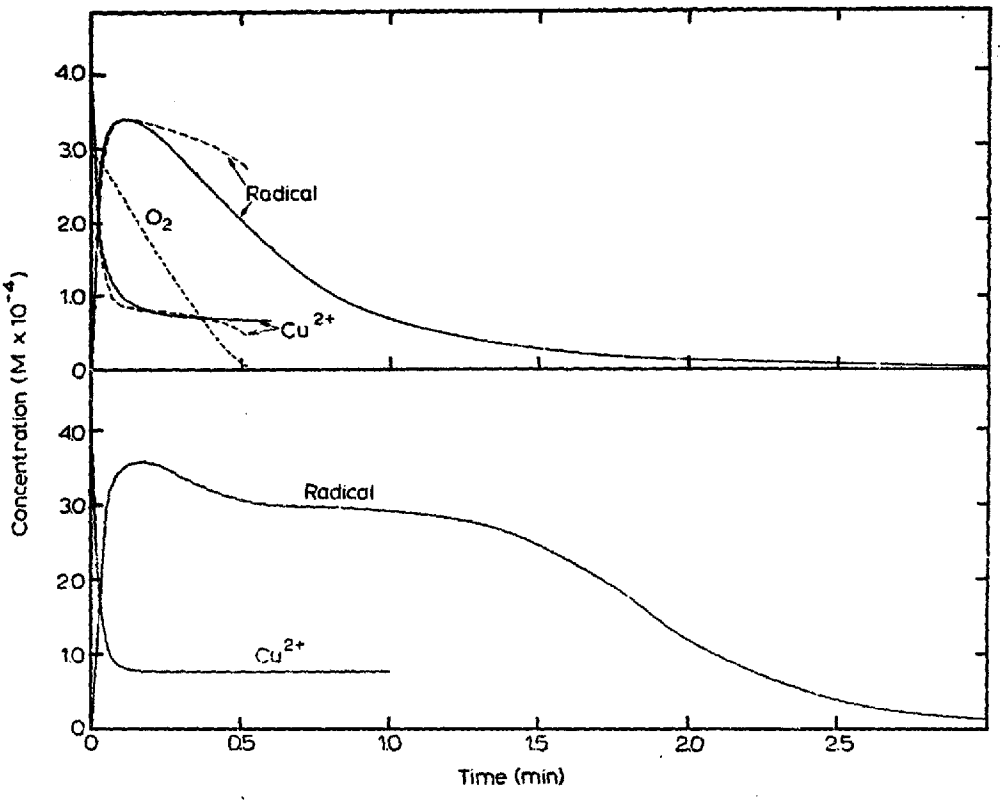


Fig. 4. Experimental (solid lines) and calculated (dashed lines) ESR absorption of $Cu^{2+}$ and of free radical in experiments with ceruloplasmin and PPD. The calculated curves are obtained with $k'_1 = 7.4 \cdot 10^3\ M^{-1} \cdot min^{-1}$, $k'_2\ h = 71\ M^{-1/4} \cdot min^{-1}$ and $k_d = 1.25 \cdot 10^4\ M^{-1} \cdot min^{-1}$ (*cf.* Eqns. 9–11). Concentrations and buffer as in Fig. 3. The upper part of the figure shows results obtained with solutions equilibrated with air; the lower part shows results when the substrate solution had been bubbled through with pure $O_2$.

TABLE I

KINETIC PARAMETERS FOR CERULOPLASMIN IN 0.1 M TRIETHYLAMINE–ACETIC ACID BUFFER (pH 5.5) AT 17°, DETERMINED BY ESR MEASUREMENTS

Initial concentrations: total copper ($c_{tot}$), $8 \cdot 10^{-4}$ M; $Cu^{2+}$ ($c_0$), $4 \cdot 10^{-4}$ M; $O_2$ ($u_0$), $2.9 \cdot 10^{-4}$ M; and substrates, $5 \cdot 10^{-3}$ M.

| *Substrate* | $T_{1/2}$ *(min)* | $c_S/c_0$ | $k_1$ *(min$^{-1}$)* | $q'_{O_2}$ *(min$^{-1}$)* | $T_s$ *(min)* | $q''_{O_2}$ *(min$^{-1}$)* | $k_2$ *(min$^{-1}$)* |
|---|---|---|---|---|---|---|---|
| PPD | 0.015 | 0.20 | 37 | 0.92 | 0.4 | 0.9 | 9.2 |
| Pyrogallol | 0.065 | 0.47 | 5.6 | 0.34 | 1.4 | 0.27 | 5.0 |
| Catechol | 0.08 | ~0.48 | 4.5 | ~0.27 | 3 | 0.12 | 4.2 |
| Quinol | 0.4 | 0.80 | 0.35 | 0.035 | 10 | 0.036 | 1.4 |
| Hydroxylamine | — | >0.7 | — | — | >20 | — | — |

was used; the sensitivity of the apparatus did not allow a sufficient slowing down of the reaction by use of lower concentrations.

The kinetics of radical formation and decay was studied with PPD. Experimental curves with ceruloplasmin, involving two different concentrations of $O_2$, are shown in Fig. 4. The radical kinetics of ceruloplasmin and laccase are compared in Fig. 5. Both figures also include calculated curves (see DISCUSSION). With DPP,

the radical signal was so strong that it was impossible to follow the decay of the $Cu^{2+}$ signal.

When a low modulation field (0.3 gauss) was used the spectrum of the radical formed with PPD was resolved into more than 75 lines. The spectrum was in all details (except possibly for a broad superimposed signal with very small amplitude) identical to that previously reported[14] from the PPD positive ion, $[NH_2C_6H_4NH_2]^+$.

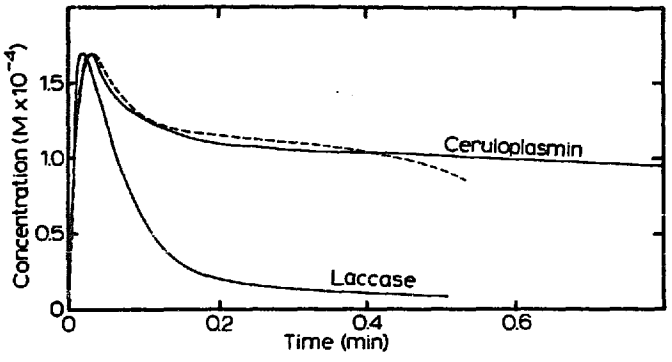


Fig. 5. ESR absorption from radicals in ceruloplasmin and laccase solutions with PPD as substrate (solid lines) and curve calculated with $k'_1 = 7.4 \cdot 10^3\ M^{-1} \cdot min^{-1}$, $k'_2 h = 71\ M^{-1/4} \cdot min^{-1}$ and $k_d = 1.0 \cdot 10^5\ M^{-1} \cdot min^{-1}$ (dashed line). Before mixing, the substrate was in 0.1 M triethylamine–acetic acid (pH 5.5), the ceruloplasmin in 0.3 M NaCl and the laccase in sodium phosphate buffer, pH 6.8, $I$ 0.05. Modulation field 21 gauss.

The line width of the components decreased slightly during the reaction and the time course of the amplitude of one of the components differed in the initial stages of the reaction from the time course of the overmodulated radical signal, as demonstrated in Fig. 6.

An ESR spectrum of a frozen solution of azurin is shown in Fig. 7, which also includes a calculated spectrum (see DISCUSSION). In one experiment, the $Cu^{2+}$ concen-

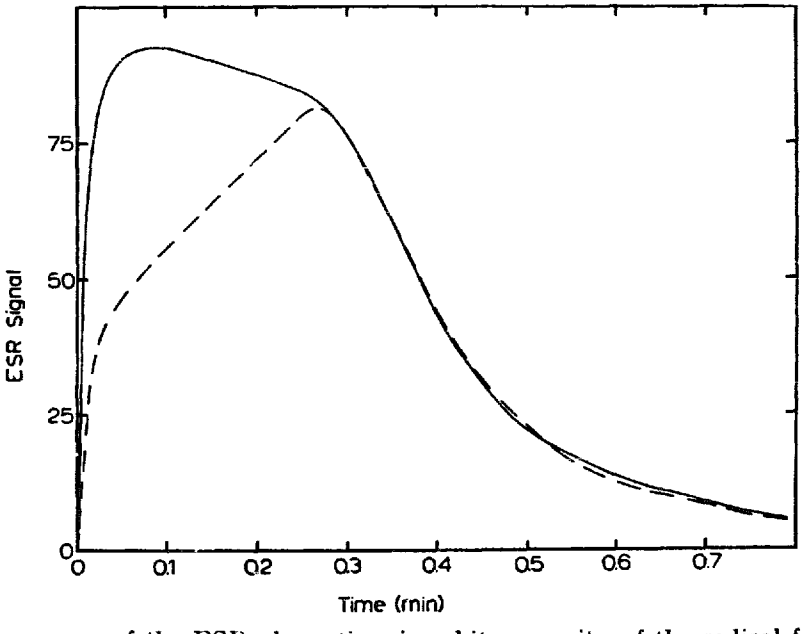


Fig. 6. Time course of the ESR absorption, in arbitrary units, of the radical formed with PPD (0.01 M) as substrate to ceruloplasmin (1 mM total copper). Modulation fields: solid line, 10 gauss; dashed line, 0.3 gauss. Buffer as in Fig. 3. The temperature was about 25°.

tration on the basis of the intensity of the ESR absorption was estimated to be $7.9 \cdot 10^{-4}$ M, while the total copper concentration, on the basis of the absorbancy at 625 mμ (extinction coefficient, $3.5 \cdot 10^3$ $M^{-1} \cdot cm^{-1}$, see ref. 8), was found to be $8.4 \cdot 10^{-4}$ M.

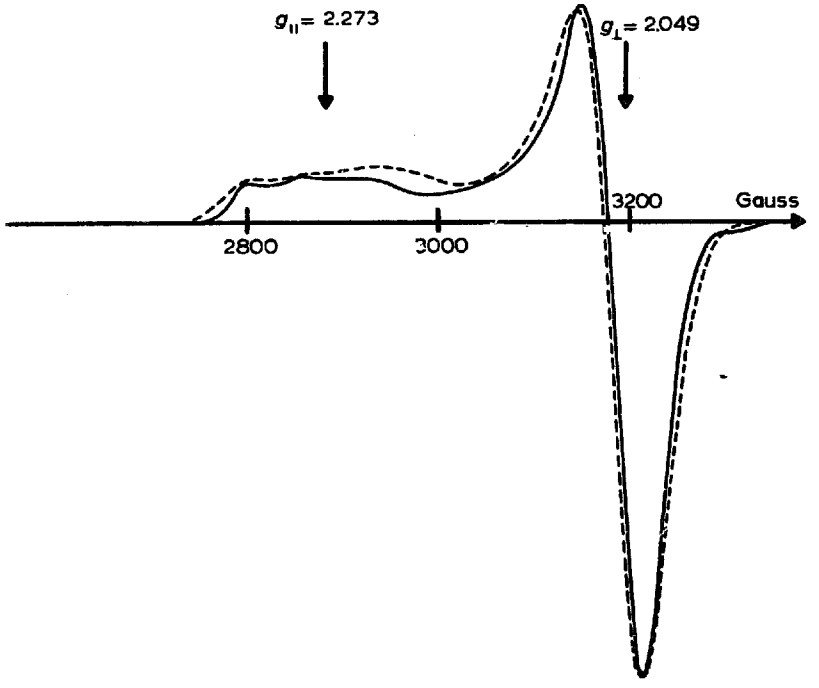


Fig. 7. Experimental ESR spectrum at 77° K of an approx. $5 \cdot 10^{-4}$ M aqueous solution of azurin solid line) and spectrum calculated with Gaussian shape (dashed line) with $g_{\parallel} = 2.273$, $g_{\perp} = 2.049$, $|A| = 55$ gauss $= 0.0^{-}6$ $cm^{-1}$, $B = 0$ and line widths $\Delta B = 49$, 56, 63 and 70 gauss for the four hyperfine lines in order with increasing magnetic field. Microwave frequency, 9166 Mcycles/sec.

## DISCUSSION

In this discussion, we will first show that the catalytic action of ceruloplasmin involves reduction of the protein-bound $Cu^{2+}$ ($ECu^{2+}$) by the substrate ($AH_2$) followed by reoxidation of the metal by $O_2$. Then some details of the enzymic reaction will be discussed.

Earlier experiments[4] have shown that almost all of the $Cu^{2+}$ signal from ceruloplasmin disappears in the presence of large excess of substrate. The residual signal, which is probably due to non-specifically bound $Cu^{2+}$, has a small amplitude. For the initial phase leading to the steady state, the $AH_2$ and $O_2$ concentrations can be regarded as constants and we have the following relation for the concentration ($c$) of $Cu^{2+}$:

$$\frac{dc}{dt} = -k_1 c + k_2 (c_0 - c) \qquad (1)$$

Subscripts o and s stand for initial and steady-state concentrations, respectively. The first term on the right of Eqn. 1 represents the reduction by $AH_2$ and the second the reoxidation by $O_2$. The possibility of reduction by an intermediate radical will be discussed below. The proportionality between rates and concentrations requires that possible enzyme–substrate complexes are formed sufficiently rapidly at the substrate concentrations used. In fact, kinetic evidence (BROMAN, unpublished)

suggests the involvement of an enzyme–$AH_2$ complex. However, the experimentally found decay of $c$ to the steady-state value $c_s$ is fairly close to the exponential decay predicted by Eqn. 1 (*cf.* Fig. 4).

In principle, we could obtain $k_1$ directly from the slope of the decay curves at time zero. Experimentally, it is easier to measure the time $T_{1/2}$. We therefore rewrite Eqn. 1 as

$$\frac{dc}{dt} = -(k_1 + k_2)\left(c - \frac{k_2 c_0}{k_1 + k_2}\right) = -\lambda (c - c_s) \qquad (2)$$

and calculate $k_1$ from

$$k_1 = \lambda (1 - c_s/c_0) \qquad (3)$$

where $\lambda$ is given by $\lambda = \ln 2/T_{1/2}$. Values of $k_1$ are found in Table I.

As $AH_2$ on the basis of equivalents is present in a 10-fold excess over $O_2$, we can take the concentration of $AH_2$ and thus also $k_1$, as constants during the steady-state period. The reduction rate is then $k_1 c_s$, which, in the steady state, must equal the reoxidation rate. As $O_2$ is reduced to $H_2O$ in the reaction, the value of $q'_{O_2}$ (Table I), defined as moles of $O_2$ consumed per mole of total copper ($c_{tot} \approx 2\, c_0$) in unit time (min) during the steady state, should be given by

$$q'_{O_2} = \tfrac{1}{4} k_1 \cdot \frac{c_s}{c_{tot}} \qquad (4)$$

The fact that the end of the steady state is so well defined (Fig. 3) shows that the reoxidation rate must be nearly independent of the $O_2$ concentration and that the $O_2$ must be almost exhausted at the end of the steady state. Thus, the $O_2$ consumption can also be estimated from the duration of the steady state ($T_s$) and the $O_2$ concentration at time zero ($u_0$):

$$q''_{O_2} = \frac{u_0}{c_{tot} \cdot T_s} \qquad (5)$$

$T_s$ has been measured to the time of exhaustion of $O_2$, as judged from numerical calculations (see Fig. 4 and below) and $u_0$ has been calculated from the solubility of $O_2$ in water. The good agreement between $q'_{O_2}$ and $q''_{O_2}$, seen in Table I*, is quite conclusive evidence that oxidations catalyzed by ceruloplasmin involve reduction and oxidation of copper. Such a rigorous test has not been possible with laccase, as $k_1$ was in all cases too large to be measured. However, this is what would be expected, as the overall rate of oxidation, based on the concentration of total copper, is of the order of 1000 times greater than that with ceruloplasmin with all substrates tested (BROMAN, unpublished).

A detailed discussion of all the steps in the enzymic reaction is of course not possible on the basis of our experiments. A few remarks may, however, be made. The reactions leading to the production of "free" free radicals may be written as

$$E Cu^{2+} + AH_2 \rightleftharpoons E Cu^{2+} AH_2 \qquad (6)$$

$$E Cu^{2+} AH_2 \longrightarrow E Cu^{1+} (AH_2)^+ \qquad (7)$$

$$E Cu^{1+} (AH_2)^+ \rightleftharpoons E Cu^{1+} + (AH_2)^+ \qquad (8)$$

* It should be noted that the oxidase activity of ceruloplasmin is not a linear function of the concentration of the protein preparation used (BROMAN, unpublished). Thus, the $q_{O_2}$ values in Table I are lower than would be expected from the specific activity determined at lower concentrations (see ref. 15).

followed by the reoxidation of $ECu^{1+}$. This could in a very formal way be described through the reaction

$$ECu^{1+} + \tfrac{1}{4}\,O_2 + H^+ \xrightarrow{k'_2} ECu^{2+} + \tfrac{1}{2}\,H_2O \qquad (9)$$

which only expresses the stoichiometry of the oxidation. The radical has been assumed to be $(AH_2)^+$ and not $AH\cdot$. This is certainly true in the reaction with PPD where no $AH\cdot$ radical could be detected. As discussed above, an enzyme–$AH_2$ complex is probably formed (Eqn. 6), and the constant $k_1$ in Eqn. 1 should then depend on the rate constants of Eqns. 6 and 7 as well as on the $AH_2$ concentration. The constant $k_2$ in Eqn. 1 should in a similar manner be a combination of the rate constants of Eqns. 8 and 9. From Eqns. 2 and 3 we have that $k_2 = \lambda \cdot c_S/c_0$ and $k_2$ is tabulated in Table I. Now, the experiments with ascorbic acid indicate that if the reaction in Eqn. 9 were the limiting step in the reoxidation, the value of $k_2$ would exceed 1500 $min^{-1}$, which certainly is much higher than the values found (see Table I). On the other hand, if Reaction 8 were rate limiting, the concentration of the complex $ECu^{1+}(AH_2)^+$ would be high. Its signal is likely to be a rather broad radical signal, as the tumbling of the protein molecules is much too slow to average out the anisotropy of the hyperfine structure. We have no evidence for any radical signal except from PPD (and DPP). With PPD as substrate, we have the difficulty of separating a broad signal with small amplitude and a well-resolved resonance structure such as the $(AH_2)^+$ signal. In Fig. 6 the time course of the overmodulated signal, which should be proportional to the total radical concentration, is compared to the time course of the $(AH_2)^+$ signal. Before the $O_2$ is exhausted, the $(AH_2)^+$ signal increases as the line width decreases with decreasing $O_2$ concentration, but the difference between the signals is quantitatively what could be expected on the basis of dipolar broadening from $O_2$. After the exhaustion of $O_2$, the signals decay almost identically, suggesting that, in this case also, the concentration of protein-bound radical is low.

In a few cases, the differential equations obtained from the equation (*cf.* Eqns. 6–8)

$$ECu^{2+} + AH_2 \xrightarrow{k'_1} ECu^{1+} + (AH_2)^+ \qquad (10)$$

together with Eqn. 9 and the equation

$$2(AH_2)^+ \xrightarrow{k_d} A + AH_2 + 2H^+ \qquad (11)$$

were integrated numerically by means of an IBM 1620 computer. Some of the results are shown in Figs. 4 and 5 (dashed lines). They illustrate that it is possible to obtain a steady state of the $Cu^{2+}$ concentration even if the $O_2$ concentration decreases to near zero. Other reaction schemes were also integrated. If instead of Eqn. 11 we used

$$ECu^{2+} + (AH_2)^+ \longrightarrow ECu^{1+} + A + 2H^+ \qquad (12)$$

no pronounced maximum in the radical concentration prior to the steady state of the radical could be obtained to correspond with that found experimentally (Fig. 5, *cf.* also the lower part of Fig. 4). Also, if Eqns. 10 and 9 were replaced by

$$ECu^{2+} + AH_2 \longrightarrow ECu^{1+}(AH_2)^+ \qquad (13)$$

$$ECu^{1+}(AH_2)^+ + \tfrac{1}{4}\,O_2 + H^+ \longrightarrow ECu^{2+} + (AH_2)^+ + \tfrac{1}{2}\,H_2O \qquad (14)$$

no such maximum in the total radical concentration appeared with the radicals decaying by dismutation or by enzyme catalysis. These results provide further evidence against the presence of high concentrations of an enzyme–product complex. Thus, the low reoxidation rate in the ESR experiments is probably not due to a slow dissociation of an enzyme–product complex. Other mechanisms, such as inhibition of the reoxidation by the substrates or their products, might be the cause of the low rate.

In considering the $O_2$ consumption we assumed that $Cu^{2+}$ was not reduced by radicals. The numerical calculations discussed above strongly suggest that this assumption is valid. The fact that a similar rate of radical decay is found with both laccase and ceruloplasmin, as seen in Fig. 5, despite the much larger overall rate and rate of copper reduction with laccase (see above), is also evidence against an enzymic decay mechanism. The steady state obtained with ceruloplasmin, but not with laccase, is due to the longer duration of the overall reaction. It should be noted that NAKAMURA[16] has previously found that the decay of semiquinone radicals produced by the catalytic action of laccase is also consistent with Eqn. 11 but not with an enzymic mechanism (for another similar case, see ref. 17).

The numerical calculations give a value for $k_d$ (Eqn. 11) of about $10^4\ M^{-1}\cdot min^{-1}$ from Fig. 4 and about $10^5\ M^{-1}\cdot min^{-1}$ from Fig. 5. The different values may be related to the different concentration of $Cl^-$ in these experiments, as BROMAN[18] has found that $Cl^-$ markedly affects the rate of colour development with PPD (*cf.* ref. 19). This would suggest that the effect observed by BROMAN[18] does not involve the enzyme reaction itself but rather the rate of radical decay.

Rapid electron transfer, as occurs with laccase and ceruloplasmin in the reaction represented by Eqn. 7, requires, among other factors, an overlap of the donor and acceptor orbitals. Thus, it should be favoured by a high degree of delocalization of the unpaired hole of the electron acceptor, $Cu^{2+}$. Such a delocalization has previously[3] been inferred from the spectral and ESR properties of these two proteins.

While the present study clearly shows that the function of $Cu^{2+}$ in these two enzymes is to undergo a reversible valency change, the role of the $Cu^{1+}$ found even in the oxidized enzymes[4,6] remains to be elucidated. BEINERT *et al.*[7] have suggested that the low hyperfine splitting observed with these two proteins, which has been used as evidence for a high degree of delocalization[3], may be due to an exchange interaction between $Cu^{2+}$ and $Cu^{1+}$. However, the ESR measurements on azurin (Fig. 7), as well as recent ESR studies by MASON[20] on the corresponding protein from Pseudomonas species, indicate that this is not a unique explanation of the spectral and ESR properties. These proteins have as high extinction coefficients (on the basis of $Cu^{2+}$ concentration) as laccase and ceruloplasmin, and show an even lower hyperfine splitting, 0.006 $cm^{-1}$, comparable to that of cytochrome oxidase[7]. However, they contain only 1 copper atom per molecule[8,20], which should exclude the possibility of exchange interaction. Furthermore, quantitative ESR measurements (see RESULTS and ref. 20) indicate that all copper is present as $Cu^{2+}$, which also speaks against interaction.

It should be noted, that the spectrum published by MASON[20] and that in Fig. 7 have in common, in contrast to the ceruloplasmin spectrum, that the widths of the hyperfine lines seem to be different. Also, even if the lines are allowed to take different widths, it has not been possible to make a good reconstruction of the azurin spectrum

(see Fig. 7) by such calculations as gave good agreement in the case of ceruloplasmin[21].

In view of the state of Cu in bacterial proteins, it would seem desirable to find an alternative function of the $Cu^{1+}$. An attractive hypothesis appears to be that the $Cu^{1+}$ should partake in substrate binding by interacting with the $\pi$-electron system of the substrates. There are at least two types of facts in favour of such an hypothesis. First, complexes involving $\pi$-electron system of the ligand are formed preferentially with transition metals in their low valencies[22]. Second, it has recently been pointed out[15] that the best substrates of ceruloplasmin are aromatic compounds with a high density of $\pi$-electrons in the ring, and there appears to be a direct relationship between the density of $\pi$-electrons in the substrate and the rate of the reaction. If substrate binding occurs in this manner, it may also result in a delocalization of the donor orbital, thus facilitating the electron transfer. We hope that further studies on substrate and inhibitor interactions with the native and modified enzymes may yield further clues to these questions. The factor responsible for the much lower specific activity of ceruloplasmin compared to laccase also remains to be elucidated. However, the great similarity in the state of $Cu^{2+}$ in these two proteins[3,4,6] suggests that this involves the donor, rather than the acceptor, properties in the enzyme–substrate complex.

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