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RADICAL SCAVENGING AND ELECTRON-TRANSFER REACTIONS IN *POLYPORUS VERSICOLOR* LACCASE

A PULSE RADIOLYSIS STUDY

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The interaction of the radicals $OH^{\cdot}$, *t*-$BuO^{\cdot}$, e_{aq}^{-}, $CO_2^{\overline{\cdot}}$ and $O_2^{\overline{\cdot}}$ with the copper oxidase, laccase, from *Polyporus*, has been studied by the pulse-radiolysis technique. Each of these radicals formed transient adducts with a broad absorption maximum around 310 nm. Analysis of the optical properties and of the very fast rates of formation of these compounds shows that each radical interacts with a limited number of sites on the polypeptide part of the protein amongst $\text{R-S}\overset{\cdot}{\text{-}}\text{S-R}$, histidine and aromatic residues. Interaction with the carbonyl group of some of the peptide bonds is also possible. The few target sites are probably hit simultaneously and electron transfer between these sites may also occur. In all cases, in a subsequent step, intramolecular electron transfer from the polypeptide radical adducts leads to a partial reduction of the blue type-1 Cu^{2+} with rates varying between 10^3 and 10^4 s^{-1}. Further reduction of the type-1 Cu^{2+} occurs through a slow intermolecular reaction between two laccase radical transient adducts. In the case of $CO_2^{\overline{\cdot}}$ and $O_2^{\overline{\cdot}}$, this slow reduction could alternatively be due to an intermolecular reaction between laccase and $CO_2^{\overline{\cdot}}$ or $O_2^{\overline{\cdot}}$. The oxidant radicals $OH^{\cdot}$, $Br_2^{\overline{\cdot}}$ and $(SCN)_2^{\overline{\cdot}}$, which formed radical adducts with fully ascorbate-reduced laccase, did not induce any type-1 copper reoxidation.

1. Introduction

Polyporus versicolor laccase (monophenol, dihydroxy-L-phenylalanine:oxygen oxidoreductase, EC 1.14.18.1) catalyzes the one-electron oxidation of substrates by coupling it to the four-electron reduction of molecular oxygen to water [1]. Attempts at understanding the mechanism of this reduction have been made in recent years, as the interactions of H_2O_2, and possibly that of $O^{\overline{\cdot}}$, with copper oxidases have been demonstrated by kinetic methods such as stopped-flow spectroscopy and rapid-freeze EPR [2–5]. While the type-1 Cu^{2+}, absorbing near 610 nm, was recognized as the primary electron acceptor from the reducing substrate, the type-3 copper site, absorbing around 330 nm, was suggested to interact, in its reduced Cu^{+}-Cu^{+} state, with molecular oxygen. Anaerobic reduction experiments on *Rhus vernicifera* laccase [2,3] indicate that reduced type-1 and -2 sites contribute one electron each to the reduction of the two-electron-accepting type-3 site. It has also been shown that H_2O_2 might interact with type-2 Cu^{2+} in *Polyporus* laccase [4] and type-3 Cu^{2+}-Cu^{2+} in *Rhus* laccase [5]. Finally, it is known that type-2 Cu^{2+} is easily accessible to water and can bind at least one water molecule [6].

A mechanism of electron transfer in *Rhus* laccase has been proposed by Andréasson and Reinhammar [3], in which $O^{\overline{\cdot}}$ was included, the product of reduction of O_2 by three electrons released from reduced type-1 and -3 coppers. Furthermore, OH^{-} was shown to interact with type-2 Cu^{2+}.

In any attempt to integrate these findings in a

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mechanistic scheme, one has to solve the following problem. Are the possible intermediates in the reduction of molecular oxygen, $O_2^{\cdot -}$, H_2O_2 and $OH^{\cdot}$, released into solution from their respective primary sites where they are formed in order to reach a secondary site where further reduction can occur? Alternatively, are these intermediates stabilized on various protein sites, not necessarily containing copper? If this were the case, could reduction take place by electron transfer between distant, structurally and functionally different sites?

The first answer to these questions was given by Faraggi and Pecht [7–10]. The pulse-radiolysis technique provides a means to produce radicals in solution within a few nanoseconds after the pulse. These radicals are available for reaction with the protein solute for times varying, according to the species, from a few microseconds for e_{aq}^- and $OH^{\cdot}$ to a few milliseconds for $O_2^{\cdot -}$, before the various reactions between these radicals are completed. In their studies of fungal laccase and human ceruloplasmin, Faraggi and Pecht [8,9] showed that e_{aq}^-, a potent reducing agent, interacts primarily with the polypeptide backbone and amino acid residues (aromatics and disulfides) to form radical adducts. In a subsequent step, electron transfers from these radicals to type-1 Cu^{2+} take place leading to the reduction of this copper atom. In contrast, they found that e_{aq}^- reduced directly, by diffusion-controlled kinetics, the single blue Cu^{2+} of *Pseudomonas aeruginosa* azurin [7]. Competing side reactions with amino acid residues of this protein also occurred.

Our aim in undertaking this work was to investigate whether Faraggi and Pecht's findings for e_{aq}^- would also apply for other radicals such as $OH^{\cdot}$, *t*-BuO$^{\cdot}$, $O_2^{\cdot -}$ and $CO_2^{\cdot -}$. We have shown, as reported here, that all these radicals formed adducts with the polypeptide part of the protein. We have carefully analyzed the kinetics of formation of these adducts in order to ascertain whether direct reduction of the type-1 Cu^{2+} could occur. We have also shown that in all cases, electron transfer took place between the radical adducts and type-1 Cu^{2+}. We have finally discussed some restrictions that need to be included in any scheme of the catalytic redox cycle of laccase.

2. Materials and methods

Laccase A was extracted from a strain of *P. versicolor*, a fungus, kindly supplied by Dr. Reinhammar (Göteborg). A large-scale culture (200 l medium) was performed according to Fåhreus and Reinhammar [11]. Laccase purifications were made following these authors, except for slight modifications. Purity was checked by measuring the absorbance ratio ($A_{280}/A_{610} \leq 15$), specific activity [11] (≥ 2000 units/mg) and by isoelectric focusing between pH 2.5 and 6 [12]; this showed that we obtained only laccase A with bands at pH 3.21 and 3.44.

Pulse-radiolysis experiments on laccase solutions in potassium phosphate (0.01 M), pH 6.0, were carried out with a Febetron 707 instrument giving 20-ns pulses of 1.8 MeV electrons. Doses were varied between 1 and 200 krad. A pulse of 10 krad in argon-saturated solutions yielded e_{aq}^- at an initial concentration of 26 μM. Radical yields were taken as $G(e_{aq}^-) = 2.63$, $G(OH^{\cdot}) = 2.72$, $G(H_2) = 0.45$, $G(H^{\cdot}) = 0.55$, $G(H_2O_2) = 0.68$, $G(H_3O^+) = 3.3$ and $G(OH^-) = 0.6$ [13]. The solutions contained in a spectrosil cell (2.5 × 2.5 × 0.25 cm) were irradiated uniformly. The spectrophotometric analysis was carried out with a standard device. The analyzing light path was 2.5 cm.

2.1. Computer analysis of data

Kinetics of radical decay or those of the formation of radical adducts to the protein were analyzed by taking into account the relevant reactions among the following (rate constants are taken from refs. 13 and 14):

$$H_2PO_4^- + e_{aq}^- \rightarrow HPO_4^{2-} + H^{\cdot} \quad (k = 4.9 \times 10^7\ M^{-1}\,s^{-1}) \tag{0}$$

$$e_{aq}^- + e_{aq}^- + 2H_2O \rightarrow H_2 + 2OH^- \quad (k = 5.4 \times 10^9\ M^{-1}\,s^{-1}) \tag{1}$$

$$e_{aq}^- + H^+ \rightarrow H^{\cdot} \quad (k = 2.35 \times 10^{10}\ M^{-1}\,s^{-1}) \tag{2}$$

$$e_{aq}^- + OH^{\cdot} \rightarrow OH^- \quad (k = 3.0 \times 10^{10}\ M^{-1}\,s^{-1}) \tag{3}$$

$OH^{\cdot} + OH^{\cdot} \rightarrow H_2O_2$

$(k = 5.3 \times 10^{9}\ M^{-1}\,s^{-1})$ (4)

$e_{aq}^{-} + H^{\cdot} + H_2O \rightarrow H_2 + OH^{-}$

$(k = 2.5 \times 10^{10}\ M^{-1}\,s^{-1})$ (5)

$OH^{\cdot} + H_2 \rightarrow H_2O + H^{\cdot}$

$(k = 4.9 \times 10^{7}\ M^{-1}\,s^{-1})$ (6)

$H^{\cdot} + H^{\cdot} \rightarrow H_2$

$(k = 1.3 \times 10^{10}\ M^{-1}\,s^{-1})$ (7)

$H^{\cdot} + OH^{\cdot} \rightarrow H_2O$

$(k = 3.2 \times 10^{10}\ M^{-1}\,s^{-1})$ (8)

$H^{\cdot} + H_2O_2 \rightarrow H_2O + OH^{\cdot}$

$(k = 9.0 \times 10^{7}\ M^{-1}\,s^{-1})$ (9)

$e_{aq}^{-} + H_2O_2 \rightarrow OH^{-} + OH^{\cdot}$

$(k = 1.2 \times 10^{10}\ M^{-1}\,s^{-1})$ (10)

In solutions saturated with N_2O (0.02 M), e_{aq}^{-} and $H^{\cdot}$ are scavenged according to:

$e_{aq}^{-} + N_2O \xrightarrow{H^{+}} N_2 + OH^{\cdot}$ $(k = 8.7 \times 10^{9}\ M^{-1}\,s^{-1})$ (11)

thus leading to $OH^{\cdot}$ as the main radical.
The presence of *t*-butanol (0.1 M) led to the formation of $t\text{-}BuO^{\cdot}$:

$t\text{-}BuOH + OH^{\cdot} \rightarrow t\text{-}BuO^{\cdot} + H_2O$ $(k = 4.2 \times 10^{8}\ M^{-1}\,s^{-1})$ (12)

When solutions contained formate (0.1 M) and N_2O, in order to obtain $CO_2^{\bar{\cdot}}$, we had to use three more equations:

$HCO_2^{-} + OH^{\cdot} \rightarrow CO_2^{\bar{\cdot}} + H_2O$ $(k = 3.5 \times 10^{9}\ M^{-1}\,s^{-1})$ (13)

$HCO_2^{-} + H^{\cdot} \rightarrow CO_2^{\bar{\cdot}} + H_2$ $(k = 5.0 \times 10^{8}\ M^{-1}\,s^{-1})$ (14)

$CO_2^{\bar{\cdot}} + CO_2^{\bar{\cdot}} \rightarrow {}^{-}O_2C{-}CO_2^{-}$ $(k = 6.9 \times 10^{8}\ M^{-1}\,s^{-1})$ (15)

When the solutions containing formate were saturated with oxygen (1.36×10^{-3} M), we also had to use the following equations:

$e_{aq}^{-} + O_2 \rightarrow O_2^{\bar{\cdot}}$

$(k = 1.9 \times 10^{10}\ M^{-1}\,s^{-1})$ (16)

$CO_2^{\bar{\cdot}} + O_2 \rightarrow O_2^{\bar{\cdot}} + CO_2$

$(k = 2.4 \times 10^{9}\ M^{-1}\,s^{-1})$ (17)

$O_2^{\bar{\cdot}} + O_2^{\bar{\cdot}} + 2H^{+} \rightarrow H_2O_2 + O_2$

$(k = 1.5 \times 10^{7}\ M^{-1}\,s^{-1})$ (pH 6.0) (18)

$OH^{\cdot} + O_2^{\bar{\cdot}} \rightarrow OH^{-} + O_2$

$(k = 1.0 \times 10^{10}\ M^{-1}\,s^{-1})$ (19)

Finally, we have added to these equations those describing in the simplest way the formation of radical complexes with the protein:

$OH^{\cdot} + \text{laccase} \xrightarrow{k_{20}} \text{laccase-}OH^{\cdot}$ (20)

$e_{aq}^{-} + \text{laccase} \xrightarrow{k_{21}} \text{laccase-}e_{aq}^{-}$ (21)

$t\text{-}BuO^{\cdot} + \text{laccase} \xrightarrow{k_{22}} \text{laccase-}t\text{-}BuO^{\cdot}$ (22)

$CO_2^{\bar{\cdot}} + \text{laccase} \xrightarrow{k_{23}} \text{laccase-}CO_2^{\bar{\cdot}}$ (23)

$O_2^{\bar{\cdot}} + \text{laccase} \xrightarrow{k_{24}} \text{laccase-}O_2^{\bar{\cdot}}$ (24)

As shown in section 3, these constants, k_{20}–k_{24}, are apparent overall rate constants. More elaborate schemes are necessary to interpret the observed kinetics. Depending on the scavengers used, we had to consider a variable number of the above reactions and solve a system of simultaneous differential equations. We have used the second integration method of Runge-Kutta [15]. As the rate constants are of very different magnitudes, we had to take a very small integration step size to simulate the reactions at the initial times, but we had to increase the integration step size as the reactions proceed with time: otherwise the calculation time would have been much too long. As a rule, the integration step size was of the order of one-hundredth of the reaction time of the fastest reaction between those still occurring at a given time in the system under study.

3. Results

The various events occurring when laccase solutions were irradiated were followed by recording the kinetics of transmission changes at wavelengths between 295 and 700 nm, on time scales varying between 50 ns and 5 s per oscilloscope grid scale unit. The modes of action of all the radicals studied here, $OH^{\cdot}$, e_{aq}^{-}, $t\text{-}BuO^{\cdot}$, $CO_2^{\bar{\cdot}}$ and $O_2^{\bar{\cdot}}$, upon laccase followed a similar pattern: firstly, the primary radicals formed transient adducts with the protein at fast rates, usually before 5 µs, as judged from differential absorption spectra in 295–500 nm range. Secondly, reduction of type-1 Cu^{2+} occurred at slower rates, probably through various mechanisms: intramolecular electron transfer be-

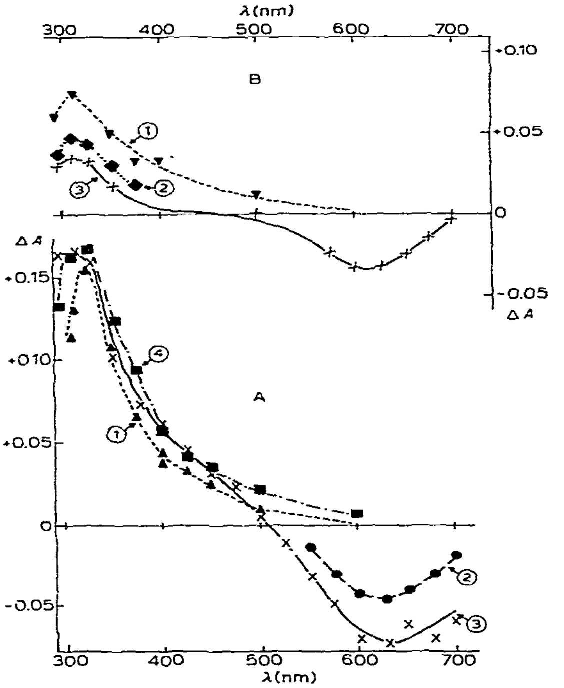

Fig. 1. (A) Interaction of laccase with $OH^{\cdot}$: difference spectra of a laccase solution (40 μM) saturated with N_2O taken 1.5 μs (1), 200 μs (2) and 250 ms (3) after the Febetron pulse. Difference spectrum of laccase (40 μM) reduced by ascorbate (160 μM) taken 200 μs after the pulse (4). Average dose: 15 krad. Average initial $OH^{\cdot}$ concentration 88 μM. (B) Interaction of laccase with t-$BuO^{\cdot}$: difference spectra of a laccase solution (40 μM) containing 0.1 M t-BuOH and saturated with N_2O, taken 5 μs (1), 2 ms (2) and 200 ms (3) after the pulse. Average dose: 15 krad. Initial radical concentration 88 μM.

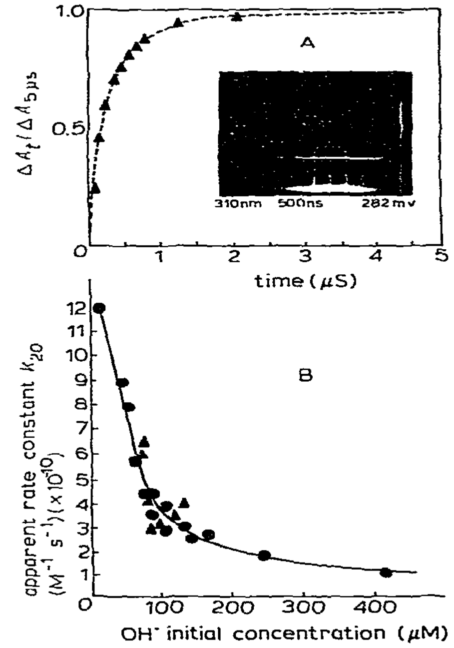

Fig. 2. (A) Kinetics of appearance of a transient adduct between laccase and $OH^{\cdot}$: variation of light transmission observed at 310 nm over 5 μs in a laccase solution (42 μM) saturated with N_2O, irradiated with a 28 krad dose. Experimental values (▲) $\Delta A_t/\Delta A_{5\mu s}$ calculated from the transmission changes. The dashed line is the ratio $[\text{laccase-}OH^{\cdot}]_t/[\text{laccase-}OH^{\cdot}]_{5\mu s}$, computed by use of eqs. 0–11 and 20 (see section 2) with $k_{20} = 2.7 \times 10^{10}\ M^{-1}\ s^{-1}$. (B) Variation of the apparent rate constant k_{20} of the overall reaction: laccase + $OH^{\cdot} \rightarrow$ laccase-$OH^{\cdot}$, observed at 310 nm with the initial $OH^{\cdot}$ concentration for laccase (45 μM) (●) and for ascorbate-reduced laccase (45 μM) (▲). Schemes involving several sites of attack of $OH^{\cdot}$ upon the protein are discussed in the text.

tween the radical adducts and type-1 Cu^{2+}, intermolecular reactions between laccase molecules and, under some conditions, between laccase and the primary free radicals $CO_2^{\cdot-}$ and $O_2^{\cdot-}$. Whenever our experimental conditions were particularly suitable, these phenomena were studied in detail.

3.1. The formation and disappearance of radical adducts in laccase solutions

3.1.1. Effect of $OH^{\cdot}$

When generated in N_2O-saturated laccase solutions, $OH^{\cdot}$ creates new absorbing species having a broad maximum around 310 nm and a shoulder in the 400–500 nm range. Fig. 1A shows the difference spectra taken 1.5 μs, 200 μs and 250 ms after the pulse, for a 15 krad average dose (initial $[OH^{\cdot}]$ 88 μM). Due to the large protein absorption no difference measurement could be made below 295 nm. The difference absorbance, measured at 310 nm, was found to reach a maximum value after 5 μs (fig. 2A). At this time and for low doses (i.e., 10 krad, or 60 μM $OH^{\cdot}$, for 40 μM laccase), the measured ΔA_{310} is roughly proportional to the dose or the initial OH$^{\cdot}$ concentration. However, at low protein concentration and large doses, the absorbance reached a plateau which could be calculated by extrapolation of the $1/\Delta A$ versus $1/[\mathrm{OH}^{\cdot}]$ plot (not shown). ΔA_{310} extrapolated to infinite dose was then found to be proportional to the laccase concentration, and attained 1.0 for a 100 μM concentration.

The observed kinetics of formation of the laccase-OH$^{\cdot}$ complex were found to be neither first nor second order under our experimental conditions ([laccase] 10–90 μM, initial [OH$^{\cdot}$] 10–400 μM). We attempted to determine the rate constant (k_{20}) of the reaction, laccase + OH$^{\cdot}$ → laccase-OH$^{\cdot}$, taking into account all the reactions of OH$^{\cdot}$ with itself and other primary radicals (eqs. 0–11). We verified that in the 295–325 nm range, OH$^{\cdot}$ absorption ($\epsilon \leqslant 100$ M^{-1} cm^{-1}) is negligible compared to that of the laccase-OH$^{\cdot}$ complex. We compared the calculated ratio $[\text{laccase-OH}^{\cdot}]_t/[\text{laccase-OH}^{\cdot}]_{5\,\mu s}$ to the measured ratio $\Delta A_t/\Delta A_{5\,\mu s}$. Good fits were obtained (fig. 2A). The apparent rate constant k_{20} was found to be extremely high. As shown in fig. 2B, k_{20} is not a true second-order constant as it varied greatly, from 12×10^{10} to 1×10^{10} M^{-1} s^{-1} when the initial OH$^{\cdot}$ concentration was varied from 10 to 400 μM, at constant laccase concentration. It did not vary noticeably with the laccase concentration. This result shows that eq. 20 is an oversimplified scheme. A scheme involving several sites of attack of the radical upon the protein will be examined in section 4.

When fully ascorbate-reduced laccase was irradiated under the same conditions as above, a radical adduct with the same spectral characteristics (fig. 1A, spectrum 4) was found to appear and disappear at the same rates (fig. 2B) as with the nonreduced enzyme.

The radical adduct laccase-OH$^{\cdot}$ is a transient species. The kinetics of disappearance appear to have several phases and to be of no definite order. The initial-phase first-order rate constant could be estimated to be of the order of 10^3 s^{-1}. Due to the small absorbance decrease, 10% of the overall differential absorbance, no precise measurement of this rate constant could be made. Full disappearance was attained only 1 min after the pulse. No attempt of detailed kinetic analysis of this phenomenon was made. Most of the radical adduct (80%) was still optically detected when maximal reduction of type-1 copper was reached, i.e., 200 ms after the pulse (see below).

3.1.2. Effect of t-BuO$^{\cdot}$

When *t*-butanol (0.1 M) is added to N$_2$O-saturated solution, OH$^{\cdot}$ is rapidly transformed to *t*-BuO$^{\cdot}$. Computation shows that the OH$^{\cdot}$ concentration is already negligible at 50 ns. The difference spectrum, taken 5 μs after the pulse, and for a 15 krad dose (initial [*t*-BuO$^{\cdot}$] 88 μM), shown in fig. 1B, indicates an interaction of *t*-BuO$^{\cdot}$ with laccase. $\Delta A_{310\,\mathrm{nm}}$ is less than half of that attained with OH$^{\cdot}$, at the same laccase and radical concentrations. The radical-protein complex is formed very quickly, before 5 μs ($k_{22} = 5 \times 10^{10}$ M^{-1} s^{-1}) and decreases slowly. Due to the small absorbance difference no attempt was made at a thorough kinetic analysis.

3.1.3. Effects of other oxidant radicals

In order to understand the mechanisms of the effects of OH$^{\cdot}$ and *t*-BuO$^{\cdot}$ on laccase (see section 4), we examined whether other oxidant radicals such as $\mathrm{Br}_2^{\cdot-}$ and $(\mathrm{SCN})_2^{\cdot-}$ interacted also with the protein. We found that they indeed did so, with rate constants 10-times greater than those of the disappearance of the radicals themselves. For example, in the presence of 40 μM laccase, $\mathrm{Br}_2^{\cdot-}$ decayed at rates varying between 1.5 and 5.1×10^{10} M^{-1} s^{-1} (initial [$\mathrm{Br}_2^{\cdot-}$] 77–21 μM), while its normal rate of decay in water is $2\text{–}6 \times 10^9$ M^{-1} s^{-1} [14]. Here again, the apparent overall rate constant increased with decreasing initial radical concentration, suggesting a multiple-site attack on the protein.

3.1.4. Effects of t-BuO$^{\cdot}$ and e_{aq}^-

e_{aq}^- and *t*-BuO$^{\cdot}$ were the primary radicals formed, at nearly equal concentration (≈ 40 μM for a 15 krad dose), when *t*-butanol was the only scavenger added. Fig. 3A shows the difference spectrum taken 200 ms after the pulse. The maximal absorbance of the transients formed upon laccase by e_{aq}^- and *t*-BuO$^{\cdot}$ is larger than that formed by *t*-BuO$^{\cdot}$ alone at a 2-fold concentration

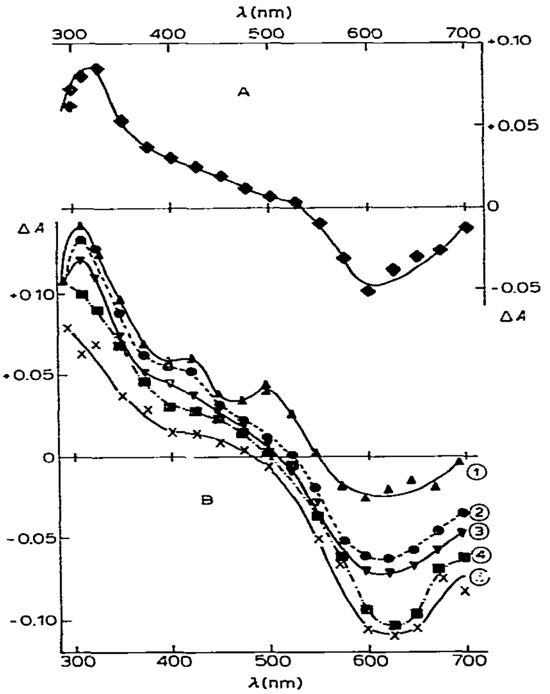

Fig. 3. (A) Interaction of laccase with e_{aq}^- + t-BuO$^\cdot$: difference spectrum of a laccase solution (40 μM) containing 0.1 M t-BuOH and saturated with argon, taken 200 ms after the pulse. Average dose: 15 krad; initial radical concentrations: t-BuO$^\cdot$ 44 μM, e_{aq}^- 40 μM. (B) Interaction of laccase with e_{aq}^- and OH$^\cdot$: difference spectra of a laccase solution (52 μM) saturated with argon taken 20 μs (1), 150 μs (2), 1.5 ms (3), 25 ms (4) and 250 ms (5) after the pulse. Average dose: 15 krad. Average initial e_{aq}^- and OH$^\cdot$ concentrations 40 μM.

(see eqs. 11 and 12). It seems therefore that e_{aq}^- adduct contribution to the absorbance is larger than that of the t-BuO$^\cdot$ adduct and smaller than that of the OH$^\cdot$-protein complex.

3.1.5. *Effect of OH$^\cdot$ and e_{aq}^-*

The cumulative effects of OH$^\cdot$ and e_{aq}^- could be detected when no scavenger was added to the aqueous solvent, both primary radicals being formed at approximately the same yield (40 μM for 15 krad). The first difference spectrum was taken 20 μs after the pulse, i.e., when the absorptions due to OH$^\cdot$ and e_{aq}^- themselves have become negligible (spectrum 1, fig. 3B).

Comparison of the spectra shown in figs. 1A (OH$^\cdot$), 1B (t-BuO$^\cdot$), 3A (t-BuO$^\cdot$ + e_{aq}^-) and 3B (OH$^\cdot$ + e_{aq}^-) shows that, at least in the 2.5–200 μs time range, each of the radicals forms a complex with the protein. It shows also that whenever two radicals are present simultaneously, their effect upon the absorbance at 310–325 nm is cumulative and that their relative contribution increases in the order t-BuO$^\cdot$, e_{aq}^-, OH$^\cdot$, in the ratio 0.4, 0.7, 1.

Two interesting features of the spectra shown in fig. 3B are worth mentioning. Firstly, the contribution in the 500 nm range is much larger when e_{aq}^- and OH$^\cdot$ interact with the protein than when OH$^\cdot$ is the only primary radical in solution. Secondly, the initial decay of this absorption band is faster ($\approx 1 \times 10^4$ s^{-1}) than that due to OH$^\cdot$ alone (fig. 1A). Actually, we found a strong dependence on wavelength of the rate constant of the initial (first 10–20%) decay of the transient absorbance: it varied monotonously between 10^4 s^{-1} at 500 nm to 10^3 s^{-1} at 300–350 nm, indicating the existence of several absorbing species. This explains the significant shifts in wavelengths as the transient adducts decay.

Due to the spectral complexity of the observed phenomena and the interference of the absorption of e_{aq}^- itself, for 5 μs after the pulse we could not analyze directly the kinetics of appearance of the complexes absorbing around 310 nm. Nevertheless, we measured the rate of disappearance of e_{aq}^- in the 500–700 nm range where e_{aq}^- has a strong absorption and determined by computation the rate constant of eq. 21 (laccase + e_{aq}^- → laccase-e_{aq}^-). As the disappearance of e_{aq}^- was over well before 10 μs after the pulse, no interference of this primary radical with the blue Cu^{2+} reduction occurred. At high protein concentration (80 μM) and small doses (1.5–3 krad or 4–8 μM e_{aq}^-), the overall decay of e_{aq}^- was found to be pseudo first order ($k = 1.2 \times 10^6$ s^{-1}; k/[laccase] = 1.5×10^{10} M^{-1} s^{-1}). The measured rate constant is 3-times larger than that measured in the absence of laccase ($k = 4.0 \times 10^5$ s^{-1}). In the computation made in order to determine k_{21}, all the necessary equations were included and the values of k_{20} were used as determined in fig. 2B. Computation yielded a rate constant k_{21} equal to 1×10^{10} M^{-1} s^{-1}, very close to the overall decay rate constant, showing that e_{aq}^-

decays mostly according to eq. 21, rather than eqs. 1–3, 5 and 10 and other equations involving $OH^{\cdot}$. For higher doses (10–20 krad), the overall e^-_{aq} decay was neither first nor second order, but an apparent rate constant k_{21} could be calculated, giving a proper fit for a given dose. It was found that k_{21} was equal to 5×10^9 M^{-1} s^{-1} for 10 krad and 1×10^9 M^{-1} s^{-1} for 17 krad. It did not vary with laccase concentration over the small range studied (40–80 μM). As in the case of $OH^{\cdot}$ and $Br_2^{\overline{\cdot}}$, k_{21} is an overall constant corresponding to an oversimplified scheme.

3.1.6. Effect of $CO_2^{\overline{\cdot}}$

The addition of 0.1 M formate to N_2O-saturated solution yields $CO_2^{\overline{\cdot}}$ as the only radical product (88 μM for 15 krad) present in solution just after the pulse (20 ns). Fig. 4A shows the difference absorption spectra taken at 200 μs and 2 ms after the pulse. As the absorbance in the ultraviolet range due to $CO_2^{\overline{\cdot}}$ itself was not negligible before 50 μs ($[CO_2^{\overline{\cdot}}] < 10$ μM or $A_{300} < 0.01$, for 15 krad doses), neither the rate of the laccase-$CO_2^{\overline{\cdot}}$ transient adduct formation nor the initial rate of its decay could be measured precisely. A lower limit for k_{23} (laccase + $CO_2^{\overline{\cdot}}$ → laccase-$CO_2^{\overline{\cdot}}$) could be estimated to be 7×10^9 M^{-1} s^{-1}. The initial rate constant of the decay of the laccase-$CO_2^{\overline{\cdot}}$ transient, estimated on the first 25% of the absorbance decrease, was found to be of the order of 10^4 s^{-1}.

3.1.7. Effect of $O_2^{\overline{\cdot}}$

The addition of 0.1 M formate to O_2-saturated solution yields $O_2^{\overline{\cdot}}$ according to eqs. 13, 14, 16 and 17 described in section 2.

Due to its slow dismutation (eq. 18), $O_2^{\overline{\cdot}}$ is, in comparison with the other primary radicals used in this study, a rather long-lived radical. 4 ms after the pulse, the absorption of $O_2^{\overline{\cdot}}$ itself was estimated to be negligible ($A_{310} < 0.005$, i.e., $[O_2^{\overline{\cdot}}] < 10$ μM for a 15 krad dose). The spectrum taken 10 ms after the pulse (spectrum 1, fig. 4B) presents a small yet significant absorption below 350 nm. There is no definite maximum in the wavelength range studied (λ ≥ 295 nm) and no absorption around 400 nm. After 500 ms, the 300 nm band has become barely detectable and a new negative broad differential absorption around 400 nm can be detected.

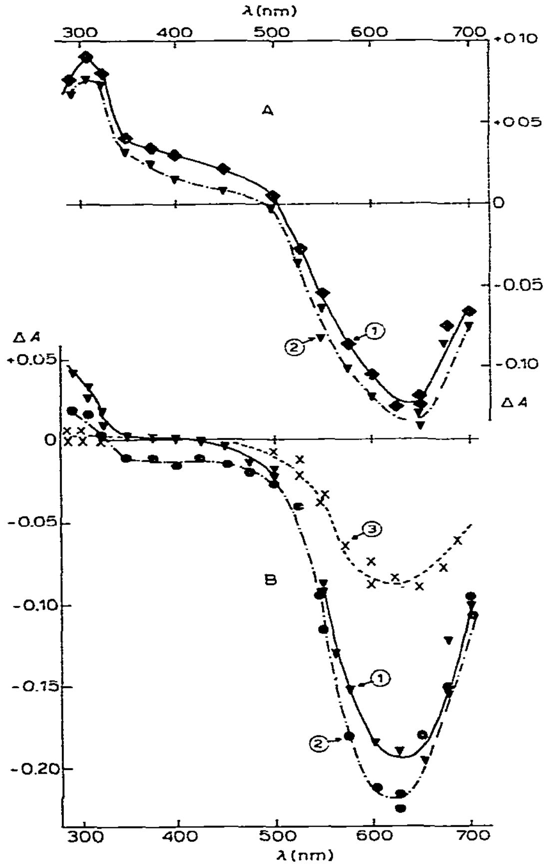

Fig. 4. (A) Interaction of laccase with $CO_2^{\overline{\cdot}}$: difference spectra of a laccase solution (42 μM) containing 0.1 M formate and saturated with N_2O taken 200 μs (1) and 2 ms (2) after the pulse. Average dose: 15 krad. Average initial $CO_2^{\overline{\cdot}}$ concentration 88 μM. (B) Interaction of laccase with $O_2^{\overline{\cdot}}$: difference spectra of a laccase solution (45 μM) containing 0.1 M formate and saturated with oxygen taken 10 ms (1) and 500 ms (2) after the pulse. Difference spectrum of the same solution containing 4 μM superoxide dismutase taken 10 ms (3) after the pulse. Average dose: 15 krad. Average initial $O_2^{\overline{\cdot}}$ concentration 88 μM.

The computation made using eqs. 1, 4, 13–21 and 23 showed that the $CO_2^{\overline{\cdot}}$ concentration was negligible (< 1 μM for 40 μM laccase and a 15 krad dose) before 1 μs, i.e., well before laccase-$CO_2^{\overline{\cdot}}$ could be formed in appreciable amounts. Therefore, we may assume that spectrum shown in fig.

4B is actually attributable to a laccase-O_2^- complex (see below for a full discussion).

As in the case of CO_2^-, only a lower limit to the rate constant k_{24} corresponding to eq. 24 (laccase + O_2^- → laccase-O_2^-) could be estimated at 2×10^6 M^{-1} s^{-1}.

In order to test the role of O_2^- in the formation of a transient complex with laccase and the reduction of type-1 Cu^{2+} (see below), we measured the difference absorption spectrum, in the presence of superoxide dismutase. We checked firstly that in the presence of superoxide dismutase (4 μM) and no laccase, the O_2^- concentration was zero at 400 μs after the pulse for 15 krad doses.

The difference spectra of laccase in the presence of superoxide dismutase (4 μM), taken between 400 μs and 10 ms, do not present any absorption in the near-ultraviolet region (spectrum 3, fig. 4B). This could be due either to the possibility that the laccase-O_2^- complex is not formed before 400 μs, in which case $k_{24} < 10^7$ M^{-1} s^{-1}, or to a dismutation, catalyzed by superoxide dismutase, of O_2^- released from the laccase-O_2^- complex.

3.2. Type-1 copper reduction

As shown in figs. 1, 3 and 4, type-1 Cu^{2+} absorbing with a maximum at 610 nm is reduced to various extents depending on the primary radical in solution. We have studied the variation in the extent of reduction with dose and the kinetics, in each case when only one primary radical was formed, $OH^{\cdot}$, CO_2^- or O_2^-. This could not be done in detail in the case of e_{aq}^-, due to mixing with $OH^{\cdot}$ or t-$BuO^{\cdot}$.

3.2.1. Levels of reduction

The differential absorbance measured around 600 nm monitoring type-1 Cu^{2+} reduction reached under our conditions a final value after 2 ms in the case of $OH^{\cdot}$ and CO_2^-, and 20 ms in the case of O_2^-. When a laccase solution was submitted to several successive pulses, the effect of each pulse on the extent of reduction was cumulative with decreasing yield. This apparent saturation effect was substantiated by studying the variation in the extents of reduction with varying pulse doses. Fig. 5 shows the results in the case of the reaction of laccase and CO_2^- taken as an example. The linearity of the $1/\Delta A_{600}$ versus 1/dose plots shows that the saturation effect is truly hyperbolic. Two useful data can be derived from these plots: the intercept with the $1/\Delta A$ axis, on the one hand, which gives the reduced laccase concentration extrapolated to infinite dose $[\text{laccase}_{red}]_{\infty}$ and the slope a on the other, the inverse of which is the initial slope for infinitely small doses in the ΔA versus dose plot. If one defines the overall yield $\bar{y}$ or efficiency of

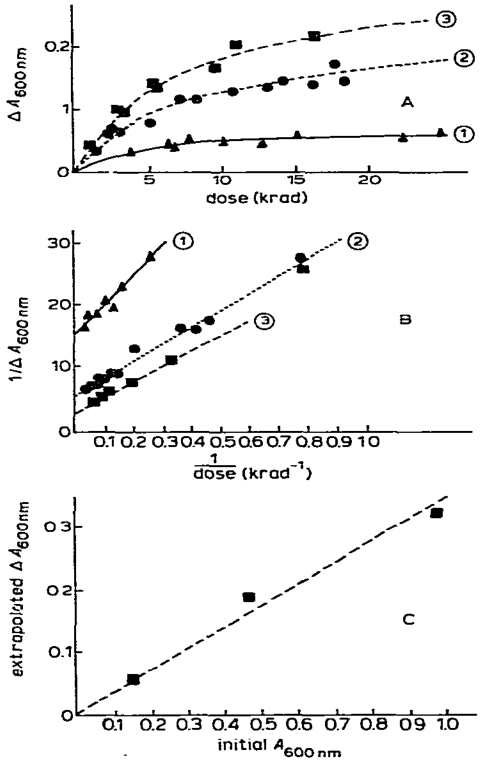

Fig. 5. Variation of the type-1 Cu^{2+} reduction level induced by CO_2^- with the radiation dose (in krad), at three different laccase concentrations: (1) 12 μM, (2) 40 μM, (3) 80 μM. (A) Saturation effect: ΔA was measured at 600 nm, 2 ms after the pulse when the type-1 Cu^{2+} reduction had attained its maximal level. (B) Reciprocal plot: the reduction efficiency or yield $\bar{y}$ (as defined in the text) is derived from the slopes a. (C) Extent of reduction for infinite dose: the ordinates (extrapolated $A_{600\,nm}$) are the inverse of the intercepts of the reciprocal plots (B) with the ordinate axis.

Table 1

Laccase type-1 copper reduction by the use of various radicals

Overall radical efficiency or reduction yield $\bar{y}$ (defined in text), reduction extent for infinite doses $[\text{laccase}_{\text{red}}]_{\infty}/[\text{laccase}]$, and reduction rates constants are given.

Radical	[laccase], (μM)	$\bar{y}$	$[\text{laccase}_{\text{red}}]_{\infty}$/ [laccase], (%)	Reduction rate constants	
				1st phase, k_1 (s^{-1})	2nd phase, $2k_2$ ($M^{-1}s^{-1}$)
$OH^{\bullet}$	20	0.43	32	$(5.5 \pm 1.4) \times 10^3$	$(2.1 \pm 0.8) \times 10^8$
	39	0.46			
	76	0.41			
$OH^{\bullet}$ (with 1 mM KF)	39	0.31	30	5×10^3	–
$t\text{-BuO}^{\bullet}$	40	0.026	10	5×10^3	–
$e^-_{aq} + t\text{-BuO}^{\bullet}$	40	0.065	14	5×10^3	–
$CO_2^{\bar{}}$	12	0.28	32	$(1.0 \pm 0.2) \times 10^4$	5×10^7
	40	0.48			
	80	0.57			
$O_2^{\bar{}}$	15	0.24	60	$(1.3 \pm 0.5) \times 10^3$	4×10^6
	37	0.30			
	71	0.44			
$O_2^{\bar{}}$ (with 1 mM KF)	21	0.48	61	$2\text{–}3 \times 10^3$	–
	83	0.44	47		
$O_2^{\bar{}}$ (with 4 μM superoxide dismutase)	24	0.043	31	$(8.2 \pm 0.9) \times 10^3$	–
	41	0.12			
	80	0.19			

the reduction of laccase by a given radical $R^{\bullet}$ as:

$$\bar{y} = \text{limit}([\text{laccase}_{\text{red}}]/[R^{\bullet}]_{\text{initial}}) \text{ for dose} \to 0$$

a simple calculation shows that:

$$\bar{y} = \frac{10^6}{\epsilon l G a}$$

where ϵ is the extinction coefficient of laccase at 600 nm (4900 M^{-1} cm^{-1}), l the light path (2.5 cm), G the radical yield and a the slope of the $1/\Delta A$ versus 1/dose plot. We found that ΔA extrapolated to infinite dose is proportional to the initial A_{600}, i.e., to the initial laccase concentration, at least in the 10–100 μM range. We could deduce that the extent of reduction of $CO_2^{\bar{}}$ for infinite doses $[\text{laccase}_{\text{red}}]_{\infty}/[\text{laccase}]_i$ is 32% of type-1 Cu^{2+}. The values similarly obtained for other radicals are listed in table 1. The yields $\bar{y}$, also listed in table 1, depended upon the nature of the radical interacting with the protein.

3.2.2. Reduction kinetics

In the cases studied, type-1 copper reduction proceeded usually through at least two kinetic phases, differing by about one order of magnitude on the time scale. For example, when laccase had interacted with $OH^{\bullet}$, the initial reduction phase was found to be first order with a rate constant $k_1 = (5.5 \pm 1.4) \times 10^3$ s^{-1}, over the range of doses (1–200 krad) and laccase concentrations (20–80 μM) used. The reduction extent of this first phase reached 90% of the overall reduction. The slower phase corresponded to a second-order reaction with a rate constant $k_2 = (2.1 \pm 0.8) \times 10^8$ M^{-1} s^{-1}. The rate constants of the reduction of type-1 Cu^{2+} by use of other radicals are listed in table 1. The first phase was always found to be a true first-order reaction. The rate constant varied by one order of magnitude, from 1.3×10^3 s^{-1} for $O_2^{\bar{}}$ to 1.0×10^4 s^{-1} for $CO_2^{\bar{}}$, depending on the primary radical used. The second phase rate constant depended more on the nature of the radical. The large standard deviations obtained are explainable by the small transmission differences, especially at small doses and small laccase concentrations.

3.3. Oxidation of type-1 copper

As already stated, oxidant radicals such as $OH^\cdot$ and t-$BuO^\cdot$ led, through the formation of a polypeptide radical intermediate, to type-1 Cu^{2+} reduction. In contrast, two other oxidant radicals, $Br_2^{\bar{\cdot}}$ and $(SCN)_2^{\bar{\cdot}}$, which interacted also with the polypeptide part of laccase did not eventually induce the reduction of type-1 Cu^{2+}. $OH^\cdot$ and $(SCN)_2^{\bar{\cdot}}$ also formed radical adducts with fully ascorbate-reduced laccase, but no reoxidation of the type-1 copper was observed. When type-1 Cu^{2+} had been reduced through the interaction of laccase with $O_2^{\bar{\cdot}}$, a very slow reoxidation occurred, taking minutes to be completed.

In all cases, when laccase had been partially bleached in the irradiation cell in the absence of oxygen, the blue color was fully restored when the solution was aerated. The optical spectrum (280–700 nm) of the reversibly reoxidized irradiated laccase was identical to that of the untreated laccase.

4. Discussion

4.1. Nature of the laccase-radical complexes

4.1.1. The transient laccase-radical complexes

Our results have shown that *Polyporus* laccase forms transient complexes with all the radicals produced in aqueous solutions that we have studied: $OH^\cdot$, t-$BuO^\cdot$, e_{aq}^-, $CO_2^{\bar{\cdot}}$ and $O_2^{\bar{\cdot}}$. In order to ascertain whether only one type of primary radical or several are responsible for the appearance of a given transient, the kinetics of its appearance had to be compared with those of the other transients by a computation including all the necessary equations among those listed in section 2.

For instance, the interaction of $CO_2^{\bar{\cdot}}$ with laccase in solutions containing formate and N_2O involves reactions between many species, such as e_{aq}^-, $OH^\cdot$, $H^\cdot$, HCO_2^-, $CO_2^{\bar{\cdot}}$ and laccase. To prove in this example that the spectra shown in fig. 4A are attributable to the interaction of laccase with $CO_2^{\bar{\cdot}}$ and with no other species, we have simulated this reaction (eq. 23), using also eqs. 1–15 and eqs. 20 and 21. The result of this calculation is as follows: laccase-e_{aq}^- and laccase-$OH^\cdot$ complexes are formed very rapidly, before 100 ns, but their concentration represents, for each complex, less than 5% of the laccase-$CO_2^{\bar{\cdot}}$ concentration. If one estimates that the laccase-e_{aq}^- and laccase-$OH^\cdot$ complexes have extinction coefficients of the order of 2–4×10^3 M^{-1} cm^{-1} at 300 nm (see the discussion below), the cummulative contribution of these species to the total ΔA would be at most 0.008. This contribution is to be compared to the $\Delta A = 0.08$ measured in fig. 4A. Therefore, the spectrum shown can be attributed, for at least 90%, to the laccase-$CO_2^{\bar{\cdot}}$ complex. A similar calculation proves that the spectrum shown in fig. 4B corresponds to a laccase-$O_2^{\bar{\cdot}}$ complex to an extend of more than 80%.

The difference spectra described here, resulting from the interaction of $OH^\cdot$ with laccase, are different from that described earlier by Andréasson et al. [2] and attributed to a protein-bound oxygen intermediate involving $O^{\bar{\cdot}}$. First, the maximal wavelength is around 360 nm instead of 310 nm, secondly the maximum absorption difference is reached 15 ms after the mixing of the fully reduced protein with oxygen. This difference shows that $OH^\cdot$ reacts with different parts of the enzyme depending on its formation procedure.

4.1.2. Nonimplication of a laccase-H_2O_2 complex

The calculations performed on the various systems under study also allow the exclusion of any significant contribution of a possible laccase-H_2O_2 complex to the ultraviolet differential spectra and to the blue copper bleaching.

In the systems yielding t-$BuO^\cdot$ or $CO_2^{\bar{\cdot}}$, the H_2O_2 concentration for a 15 krad dose is 10 μM and remains constant with time. In other systems ($OH^\cdot$, $OH^\cdot + e_{aq}^-$, $O_2^{\bar{\cdot}}$) it increases at various rates from 10 to 60 μM. ΔA_{300} and ΔA_{600}, shown in figs. 1, 3 and 4, show no relationship with the calculated H_2O_2 concentration. Furthermore, although it has been demonstrated that H_2O_2 reacts with *Polyporus* laccase [4], the compound formed has optical and kinetic properties that differ strikingly from those examined here. In fact, the laccase-H_2O_2 complex is formed in more than a few milliseconds and its maximal absorption is found at 400 nm ($\epsilon = 10^3$ M^{-1} cm^{-1}) [4]. It is stable for minutes and within the time of its existence the protein blue color is reversibly bleached. Finally, this laccase-H_2O_2 complex can be obtained only for H_2O_2/laccase ratios between 3 and 100, while in our study this ratio is between 0.3 and 1.

4.1.3. Parts of the fungal laccase involved in its scavenging properties

Comparison of the measured overall apparent rates of formation (eqs. 20–24) of the laccase-radical complexes with the rates of reactions of those radicals between themselves or with the scavengers N_2O, *t*-BuOH, O_2 (eqs. 0–19) shows that the reactions with laccase are always as fast or even faster than any other. In this sense, laccase can be considered as an efficient radical scavenger. This property seems to be quite widespread among proteins, in particular, blue copper proteins (for reviews see refs. 17 and 18; and also ref. 19). The kinetic and spectral properties described in this paper allow us, by comparing them with those of radical complexes with amino acids and peptides [17], to suggest which parts of the laccase protein could be responsible for its scavenging properties.

$OH^{\cdot}$ forms adducts with or abstracts hydrogen atoms from several types of amino acid residues tyrosine, tryptophan, histidine, cystine and cysteine at rates of the order of 1×10^{10} M^{-1} s^{-1} (see review in ref. 17) which are comparable to k_{20}. The absorption maximum situated at 310 nm (fig. 1A) enables us to limit our choice to the first three whose complexes with $OH^{\cdot}$ have maxima between 290 and 320 nm ($\epsilon \approx 2250$–3000 M^{-1} cm^{-1}) and absorb much less around 400–420 nm ($\epsilon \approx 1400$–2000 M^{-1} cm^{-1}). As ΔA_{310} extrapolated to infinite dose has been found to vary linearly with laccase concentration and reaches 1.0 for 100 μM laccase and 2.5 cm light path, we can calculate an experimental $\Delta\epsilon_{310\,nm} = 4000$ M^{-1} cm^{-1}. Therefore, we can infer that only a very limited number of residues, one or two, amongst histidine, tryptophan and tyrosine side chains can form an adduct with $OH^{\cdot}$.

As the absorption in the 400–500 nm region of fig. 1A is rather small and the decay of the absorption throughout the 295–500 nm range occurs at the same rate, there is no need to assume any significant contribution of a cysteine or cystine bridge complex with $OH^{\cdot}$. The same argument can be used for the interaction of peptide bonds with $OH^{\cdot}$: due to its characteristics ($k = 2 \times 10^9$ M^{-1} s^{-1}, $\lambda_{max} = 350$ nm, $\epsilon_{max} = 1500$ M^{-1} cm^{-1}), it can give only a small contribution to the spectra of fig. 1A.

The fastest overall rate constant (k_{21}) we have measured for the interaction of *Polyporus* laccase with e_{aq}^- is 8-times slower than that measured by Pecht and Faraggi [8] ($k = 8.5 \times 10^{10}$ M^{-1} s^{-1}). However, this apparent discrepancy can be understood, as our experimental conditions (initial $[e_{aq}^-]$ between 5 and 44 μM) were different from theirs ($[e_{aq}^-] = 0.5$ μM) and as we have shown that the apparent overall rate constant k_{21} increases with decreasing initial e_{aq}^- concentration. These values of k_{21} (10^9–8×10^{10} M^{-1} s^{-1}) are comparable to the rate constants of reactions of e_{aq}^- with R-S-S-R groups, with cysteine and histidine residues (1×10^9–3×10^{10} M^{-1} s^{-1}) [17]. It is also comparable to the rate of reaction with several (ten or more) peptide bonds to form e_{aq}^--carbonyl adducts. In fact, as shown by Tal and Faraggi [20], the observed rate constant is the sum of several rate constants, each involving a carbonyl group ($k_i = 1$–3×10^8 M^{-1} s^{-1}). As for the other amino acids, their rate constants of interaction are much smaller (10^7–10^8 M^{-1} s^{-1}) [17].

Some more comments can be made from the analysis of the spectra of figs. 1, 3 and 4. When the contribution of laccase-*t*-$BuO^{\cdot}$ shown in spectrum 3 of fig. 1A is subtracted from the spectrum of fig. 3A, the result gives the spectral contribution of the interaction of e_{aq}^- with laccase. It has a maximum at 310 nm and a quite pronounced shoulder above 400 nm. In the same way in fig. 3B, one has to bear in mind that both laccase-e_{aq}^- and laccase-$OH^{\cdot}$ contribute to it. If the contribution of laccase-$OH^{\cdot}$ in the 310 nm region is greater than that of laccase-e_{aq}^- by 40%, in the 400–500 nm region, on the contrary, it is the contribution of laccase-e_{aq}^-

that dominates largely through broad bands at 410 and 500 nm. Due to the very large absorption of (R-S-S-R)$^{\cdot}$ at 410 nm ($\epsilon = 10^4$ M^{-1} cm^{-1}) [17] only one, if any, of these groups can be formed in our case. The absorption of the imidazolium-e_{aq}^- adduct ($\epsilon_{290} = 2$–5×10^3 M^{-1} cm^{-1}) allows for one or two, at most, of these complexes on laccase. As for the peptide groups, the large ϵ value of the e_{aq}^--carbonyl adduct at 300 nm (≈ 2000 M^{-1} cm^{-1}) allows for no more than two of these groups contributing to ΔA at saturating doses. However, this is in contradiction with the estimate based on the reaction rates which give ten or more peptides bonds interacting with e_{aq}^-.

It is difficult to give any detailed structural interpretation of the spectra shown in fig. 4A, as very few data are at hand about the interaction of $CO_2^{\cdot -}$ with amino acids and polypeptides. $CO_2^{\cdot -}$ reacts with the disulfide bridge ($k = 5.6 \times 10^8$ M^{-1} s^{-1}, $\lambda_{max} = 405$ nm, $\epsilon_{max} = 10300$ M^{-1} cm^{-1} at pH 6.1) [21]. It is also known that $CO_2^{\cdot -}$ is unable to reduce imidazole and react with the peptide bond [17]. However, our spectrum (fig. 4A) has a definite maximum around 310 nm, with a shoulder around 400 nm. So (R-S-S-R)$^{\cdot}$ cannot be the only product of the interaction of laccase with $CO_2^{\cdot -}$. There must be another site as a target for $CO_2^{\cdot -}$.

Finally, though t-BuO$^{\cdot}$ and $O_2^{\cdot -}$ both form adducts with laccase, we are unable to suggest any definite site of interaction of these radicals on the protein. We must, however, emphasize the fact that these two radicals do interact with the protein, contrary to what is usually assumed [8,18,22].

In order to understand the large decrease in the apparent rate constant k_{20} with increasing initial OH$^{\cdot}$ concentration (fig. 2B), we attempted to fit our data $\Delta A_t / \Delta A_{5\mu s}$ with schemes more adequate than eq. 20. Three reasonable assumptions could be made: firstly, several types of sites on the laccase molecule could interact simultaneously with OH$^{\cdot}$ at different rates. Secondly, the various products formed could have different extinction coefficients. Thirdly, fast first-order reactions could occur between sites. These assumptions were tested on the following scheme:

$$\text{laccase}_1 + \text{OH}^{\cdot} \xrightarrow{k_{20}^1} \text{laccase}_1\text{-OH}^{\cdot} \xrightarrow{k_{20}^3} \text{laccase}_3\text{-OH}^{\cdot}$$

$$\text{laccase}_2 + \text{OH}^{\cdot} \xrightarrow{k_{20}^2} \text{laccase}_2\text{-OH}^{\cdot}$$

We added four variables to be fitted: n_1 and n_2 being the number of sites of laccase$_1$ and laccase$_2$ types, respectively, and α and β the ratios of the extinction coefficients of the products laccase$_2$-OH$^{\cdot}$ and laccase$_3$-OH$^{\cdot}$ to that of laccase$_1$-OH$^{\cdot}$. In testing the various schemes we restricted ourselves to the only values that we estimated reasonable, i.e., n_1 and n_2, integers between 1 and 4, and α and β between 0.5 and 2 (see the above discussion). One solution giving proper fits for the various *initial OH$^{\cdot}$ concentrations used (10–400 μM, see* fig. 2B) was:

$k_{20}^1 = 4 \times 10^{10}$ M^{-1} s^{-1}, $n_1 = 1$,

$k_{20}^2 = 1.5 \times 10^{10}$ M^{-1} s^{-1}, $n_2 = 3$, $\alpha = 1$,

$k_{20}^3 = 1 \times 10^5$ s^{-1}, $\beta = 1.5$.

Other solutions were found, for example, with $k_{20}^3 = 0$ or with $\alpha \neq 1$. We cannot exclude the possibility that other numerical solutions could exist within this basic scheme. Nevertheless, the solutions given here are within the range of reasonable values and show without ambiguity that several sites on the protein interact simultaneously with the radical.

4.2. *Type-1 copper reduction and electron transfer*

4.2.1. *Reduction yield*

An obvious finding of this study is that type-1 copper reduction is induced by interaction of laccase with oxidant radicals as well as with reductants. In fact, OH$^{\cdot}$ is a strong oxidant ($E_0 = +2$ V), while e_{aq}^- is a strong reductant (-2.7 V), $CO_2^{\cdot -}$ a milder reductant (-1.1 V) [16] and $O_2^{\cdot -}$ can be an oxidant at neutral pH ($E_0' = +0.95$ V) as well as a reductant (-0.33 V). Even t-BuO$^{\cdot}$, usually considered as a rather inert radical, can lead to Cu^{2+} reduction. In contrast, $Br_2^{\cdot -}$ and $(SCN)_2^{\cdot -}$, two oxidant radicals that we have shown to interact with laccase, did not induce any type-1 Cu^{2+} reduction. Strangely enough, e_{aq}^- has a poor yield or efficiency in reducing the type-1 Cu^{2+} while the other radicals have similar yields ranging from 0.30 to 0.50. The same is true for the extent of reduction for infinite dose. If one considers that the overall yield may correspond to at least two sequential events, namely the radical scavenging and the electron transfer, one can explain the difference in the yields of the effect of e_{aq}^- and $OH^\cdot$ on laccase by comparing the overall rate constants k_{20} and k_{21} to those of eqs. 1 and 4. The apparent rate constant k_{21} (1×10^9–1×10^{10} M^{-1} s^{-1}) is of the same order as the rate constant of e_{aq}^- over itself (5.4×10^9 M^{-1} s^{-1}), while k_{20} (1.2×10^{10}–1.2×10^{11} M^{-1} s^{-1}) is roughly 10-times greater than that of $OH^\cdot$ over itself (5.3×10^9 M^{-1} s^{-1}). Therefore, the relative scavenging efficiency of laccase for $OH^\cdot$ is much greater than that for e_{aq}^-. O_2^-, which has similar yields to those of $OH^\cdot$ and $CO_2^{\bar{}}$, gives a reduction extent for infinite dose of as high as 60%. This could be due to the rather long lifetime of $O_2^{\bar{}}$. When catalytic amounts of superoxide dismutase were added, which rendered the $O_2^{\bar{}}$ concentration zero within 400 μs, the ratio $[\text{laccase}_{red}]_\infty/[\text{laccase}]_i$ diminished from 60 to 31%. Under these conditions, the yield appears smaller (0.043–0.19 instead of 0.24–0.44) because the dismutation of $O_2^{\bar{}}$ catalyzed by superoxide dismutase diminished the amount of radical available for interaction with laccase.

The yields which we have measured are usually much greater than those mentioned by other authors [18,22]. We feel fairly confident about our values as they are derived from the hyperbolic saturation effects that we have demonstrated. In fact, all the points shown in fig. 5 in a given $1/\Delta A$ versus 1/dose plot contribute to the determination of the yield. In contrast, the use of only a few points corresponding to very small doses in the ΔA versus dose plot would lead to very high uncertainty in the determination of yields.

4.2.2. *Reduction kinetics*

Another of our findings, namely, that the first, fastest, phase of the reduction of type-1 Cu^{2+} is first order whatever the primary or secondary radical involved (e_{aq}^-, $OH^\cdot$, t-$BuO^\cdot$, $CO_2^{\bar{}}$, $O_2^{\bar{}}$) is in disagreement with the report of Pecht and Faraggi [8] that the reduction of fungal laccase by e_{aq}^- is a second-order process. However, it is in agreement with their finding that the reduction of ceruloplasmin by e_{aq}^- ($k = (9 \pm 1) \times 10^2$ s^{-1}) [9] and of laccase (biological source not mentioned) by $O_2^{\bar{}}$ ($k = 4.1 \times 10^4$ s^{-1} for oxygen-saturated solutions) [22] is a first-order process. The reduction of type-1 Cu^{2+} by intramolecular electron transfers from the protein site which forms an adduct complex with the primary or secondary radical to the type-1 copper site seems, therefore, to be quite a general process in the case of blue oxidases.

The measured rate constants of the first phase of reduction (5×10^3 s^{-1} for $OH^\cdot$ or e_{aq}^-, 10^4 s^{-1} for $CO_2^{\bar{}}$) corresponded roughly to the very initial rate constants measured for the decay of the laccase-$OH^\cdot$, laccase-e_{aq}^- or laccase-$CO_2^{\bar{}}$ adducts. However, these rate constants could not be measured precisely and corresponded only to 10–20% of the overall decay of these adducts. It must be emphasized that the reduction by intramolecular electron transfer can only proceed from the laccase radical adducts as the primary radical species such as $OH^\cdot$ or e_{aq}^- have totally disappeared at the time when the reduction starts (≈ 20 μs for $OH^\cdot$ or e_{aq}^-).

We must emphasize, from the following arguments derived from the mode of action of oxidant radicals, that the type-1 Cu^{2+} reduction proceeds through an electron transfer and not from a radical transfer. Firstly, $OH^\cdot$ can form an addition complex with a histidine, tyrosine or tryptophan residue. In a second step, it can abstract a hydrogen atom. In a third step, an electron can be released and migrate from this reductant radical to Cu^{2+}. Secondly, $Br_2^{\bar{}}$ and $(SCN)_2^{\bar{}}$ react also with aromatic side chains, but as they cannot abstract hydrogen atom, these radicals cannot transfer electrons.

We have no definite mechanism to propose for this intramolecular electron transfer. Electron migration could proceed by some site hopping between aromatic amino acid residues or along the polypeptide backbone. Another mechanism could be an electron tunnelling between the radical adduct site to the type-1 copper site. Unfortunately, we have no data on the distance between those sites and therefore we cannot test any of these mechanisms.

A second phase of reduction was found to occur at a rate roughly 10-times slower than the first. It accounted for a small portion (≈ 10%) of the overall reduction. When this slower phase could be fully analyzed, as was the case with $OH^\cdot$, it was shown to be a second-order reaction. Computation showed that the $OH^\cdot$ concentration was actually negligible when Cu^{2-} reduction occurred. The second-order reaction seems therefore to be an intermolecular reaction between two laccase molecules, both carrying a reducing radical on one of their amino acid residues. In such cases, the electron transfer would occur from the reduced residue of one protein molecule to the type-1 Cu^{2+} of another laccase molecule. Our value of the rate constant measured for this second phase agrees with that found for the overall reaction by Pecht and Faraggi [8] who did not observe a fast phase.

In the case of $CO_2^{\cdot-}$ and $O_2^{\cdot-}$, computations showed that during this second phase of reduction, these radicals were at high enough concentrations to contribute to a direct reduction of Cu^{2+}. However, the intermolecular process between two laccase molecules that we have just described remains a plausible mechanism.

The experiments described here and those reported by Pecht and collaborators [8–10, 17, 18] demonstrate that a limited number of amino acid residues are the first sites of the enzyme to be reduced. The second redox site to be reduced is the type-1 Cu^{2+}. Our results do not suggest any involvement of the type-3 copper pair site in the reduction process. Any such involvement, if it does occur, might be overshadowed by the large absorptions of the protein radical adducts. On the other hand, the absence of effect of F^- (1 mM) on the yield, extent and rate of reduction of type-1 Cu^{2+} induced by $OH^{\cdot}$ or $O_2^{\cdot-}$ (table 1) would argue against any implication of the type-2 Cu^{2+} site in the primary reduction steps, since F^- is known to bind to the type-2 Cu^{2+} site. However, experiments in which the type-2 copper could be directly monitored, for example, γ-radiolysis experiments coupled to EPR, such as those performed by Zgirski et al. [23], could give precise data in that respect and could lead to a better understanding of the electron-transfer process.

Finally, of the two unstable intermediate species that could be produced in the oxidase enzymatic cycle, $OH^{\cdot}$ and $O_2^{\cdot-}$, the latter alone has a lifespan sufficient for this species to be hopping from one site to another. $OH^{\cdot}$ can only act in an intramolecular electron-transfer process in the redox cycle of the enzyme.

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