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Fast Reduction of a Copper Center in Laccase by Nitric Oxide and Formation of a Peroxide Intermediate

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Abstract: The rapid reduction of one of the copper atoms (type 2) of tree laccase by nitric oxide (NO) has been detected. Addition of NO to native laccase in the presence of oxygen leads to EPR changes consistent with fast reduction and slow reoxidation of this metal center. These events are paralelled by optical changes that are reminiscent of formation and decay of the peroxide intermediate in a fraction of the enzyme population. Formation of this species is only possible if the trinuclear copper cluster (type 2 plus type 3) is fully reduced. This condition can only be met if, as suggested previously, a fraction of the enzyme contains both type 3 coppers already reduced before addition of NO. Our data are consistent with this assumption. We have suggested recently that fast reduction of copper is the mechanism by which NO interacts with the oxidized dinuclear center in cytochrome *c* oxidase. The present experiments using laccase strongly support this view and suggest this reaction as a general mechanism by which copper proteins interact with NO. In addition, this provides an unexploited way to produce a stable peroxide intermediate in copper oxidases in which the full complement of copper atoms is present. This enables the O−O scission step in the catalytic cycle to be studied by electron addition to the peroxide derivative through the native electron entry site, type 1 copper.

Introduction

Nitric oxide is known to act as a signal transducer in neurotransmission[1] and vasodilation[2] by interacting with iron in the heme protein guanylyl cyclase. However, nitric oxide may also exert potent physiological effects through interaction with copper. We have recently reported very fast reactions between nitric oxide and cytochrome *c* oxidase,[3,4] and we have explained these through a mechanism in which NO acts as a one-electron reductant of the enzyme. We have proposed that reduction occurs at Cu_B(II), the oxidized copper in the dinuclear center, and that, once reduced to Cu_B(I), this rapidly equilibrates with other redox centers in the enzyme. This rapid interaction of NO with oxidized copper has been suggested to play a key role in the control of the activity of cytochrome *c* oxidase[4] and is possibly relevant to the control of a number of other copper containing enzymes. However, the evidence for the reaction of NO with this metal is indirect and only inferred from the observed electron transfer to other redox sites.

We have tested this model using laccase (*p*-diphenol:dioxygen oxidoreductase, EC 1.10.3.2), the simplest of the family of the multicopper oxidases, which catalyzes the four-electron reduction of dioxygen to water. Laccase contains four copper atoms, classified mainly according to their EPR features:[5] type 1 (T1) or blue, which has a small parallel hyperfine coupling ($A_{\|} \sim (40-70) \times 10^{-4}$ cm^{-1} or 43−75 G), type 2 (T2) with normal EPR features, and a dinuclear EPR-undetectable site termed type 3 (T3). Optically, T1 has a relatively strong band in the visible region ($\epsilon_{615} \sim 5700$ M^{-1} cm^{-1}), T3 absorbs at 330 nm ($\epsilon_{330} \sim 3600$ M^{-1} cm^{-1}),[6] whereas T2 is practically undetectable. Electron entry from the physiological substrate into the enzyme is at T1, which transfers electrons to a trinuclear copper center formed by T2 and T3.[7,8] It is at this center, analogous to the trinuclear center found in ascorbate oxidase[9] or ceruloplasmin,[10] where oxygen binds and is reduced.

The understanding of the function of this enzyme has been greatly improved by the use of two derivatives. One of these is type 2 depleted (T2D) laccase, in which one of the copper atoms

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(T2) is removed by a chelator.[11] Studies using this derivative showed that, in contrast to the oxygen carrier hemocyanin, the dinuclear T3 reduced site in laccase does not bind oxygen[12] in contrast with previous reports.[13] A second derivative[14] is T1Hg, in which the copper atom in the T1 center has been substituted by the redox-inactive Hg(II). Using this method, it is possible to obtain an enzyme reduced with only three electrons, all of them in the trinuclear center. The combination of fully reduced (three electrons) T1Hg with oxygen results in the formation of a species which is described as a hydroperoxide, bridged between an oxidized T3 copper and reduced T2.[12,15] This intermediate has been suggested by these authors to precede the formation of the native intermediate[8] in the normal catalytic cycle.

The reaction of NO with tree laccase has been studied previously.[16] In these experiments, performed in the absence of oxygen and under a saturating NO concentration, the enzyme was fully reduced in a process that was reported to be very slow ($t_{1/2} \sim 70$ min). These experiments, however, were made with a low temporal resolution (e.g., the first EPR measurement was collected 5 min after the addition of NO). On the other hand, the redox state of type 2 copper cannot be assessed optically and EPR measurements are complicated by changes in the redox potential of the metal centers upon freezing the samples.[16] Therefore, an accurate measurement of the level of reduction of type 2 copper in the first seconds was not given. In the present work, we present evidence that NO can reduce T2 copper in laccase in a very fast reaction ($t_{1/2} < 1$ s).

Experimental Section

Laccase was obtained and purified according to the method of Reinhammar[17] from acetone powder of Rhus vernicifera (Saito & Co. Ltd. Tokyo, Japan). The concentration of laccase was determined using $\epsilon_{614} = 5700\ M^{-1}\ cm^{-1}$.[6] Static spectra were collected with a Cary 5E UV−vis−near-IR spectrophotometer. The experiments were performed at 20 °C in 0.1 M HEPES (*N*-[2-hydroxyethyl]piperazine-*N*′-[2-ethanesulfonic acid]) at pH 6 or pH 7.4. The pH was altered by passage through a Sephadex G-25 column equilibrated with buffer at the desired pH. Anaerobic laccase was prepared by degassing a solution in a cuvette presealed with a rubber cap. This was purged with N_2 gas, and after a few cycles the sample was immediately used.

Stopped-flow experiments were performed using a SX-18MV stopped-flow apparatus (Applied Photophysics, Leatherhead, U.K.). Laccase incubated anaerobically with NO was rapidly mixed with buffer containing oxygen at known concentrations. This was obtained by mixing anaerobically the degassed solution with the required volume of oxygen equilibrated buffer.

EPR spectra were measured on a Bruker EMX spectrometer with an ER 041XG microwave bridge (X-band). An ER 4122SP cavity was used. The temperature was controlled using an Oxford Instrument helium system. A Bruker WINEPR (v. 2.11) package was used for spectral analysis. Aliquots of the enzyme or reaction mixture were placed in 3 mm i.d. EPR "precision" tubes (Wilmad PQ) and frozen in ethanol thermostated in dry ice. Once frozen, the tubes were transferred to liquid nitrogen where they were stored until use. The oxidation of the type 2 copper was monitored using the intensity of the low-field component[18] at $g = 2.47$, measured using conditions in which the signal-to-noise ratio was maximized (i.e., averaging many scans at low temperature (8 K)). After the addition of NO, the band shape of this component was slightly different from the control, although it was unchanged thereafter. Due to this fact, the average intensity corresponding to the samples after 1 h was taken as 100% type 2 copper oxidation.

Nitric oxide was obtained from a Kipps apparatus maintained in a certified hood (due to the potential toxicity of NO gas). Sulfuric acid (1 M) was mixed with sodium nitrite. The gaseous product(s) of this reaction were passed through a series of traps (NaOH, H_2SO_4, H_2O, KI, and dry ice) to remove nitrogen oxides other than NO. Finally NO was collected in a gastight syringe and injected into an anaerobic solution. The NO concentration was measured with an NO electrode (Iso-NO Mark II, World Precision Instruments). The electrode was precalibrated by the addition of a standard sodium nitrite solution to excess acidified potassium iodide; this generates NO stoichiometric to the added nitrite. Additions of NO were made using a gastight Hamilton syringe.

It should be remembered that on exposure to air NO reacts spontaneously and rapidly to form toxic NO_2 gas. Due care should thus be exercised when handling NO, and all transfers should be undertaken in a fume hood.

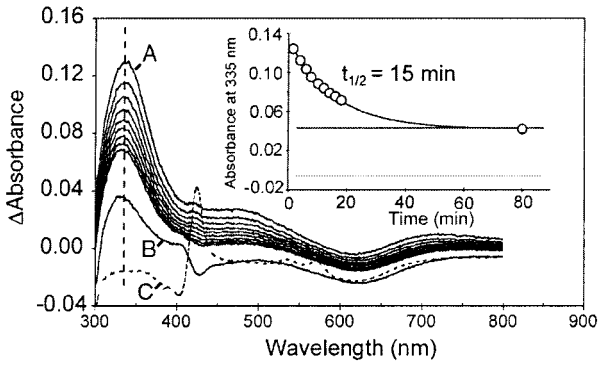

Figure 1. Spectral changes after addition of NO to laccase as prepared in the presence of oxygen. Difference spectra relative to the resting oxidized enzyme (as prepared) after the addition of an aliquot of 2 mM NO (final concentration ~ 100 μM) to a solution containing ~70 μM laccase in 100 mM HEPES, pH 6.0. The first spectrum (A) was collected immediately after the addition of NO, and spectra were collected every 2 min thereafter. Spectrum B corresponds to the sample after 80 min. A vertical dotted line is plotted to indicate the blue shift of the band as the intermediate decays. Addition of NO to an anaerobic sample in the same conditions generates spectrum C. Insert: Time course followed at 335 nm (○) fitted to an exponential decay ($t_{1/2} \sim 15$ min) (—). The features of spectrum C at 400−425 nm and the small bands in the visible region (530 and 565 nm) correspond to the formation of a ferric−NO complex of lacquer peroxidase,[19] a contaminant (less than 0.5% of the laccase concentration) that is common in these preparations. These features decayed rapidly at pH 6 ($t_{1/2} \sim 30$ s) and were not present at the time points shown (spectrum A and below). The trough at ~420 nm at 80 min (spectrum B) is caused by a further blue shift of the Soret band with respect to the oxidized enzyme.

Results

Addition of NO to resting oxidized tree laccase (as prepared) in the presence of oxygen resulted in rapid spectral changes depicted in Figure 1 (spectrum A). This difference spectrum shows prominent positive features at 335 nm, 470 nm, and a trough at 610 nm. These positive features are strongly remi-

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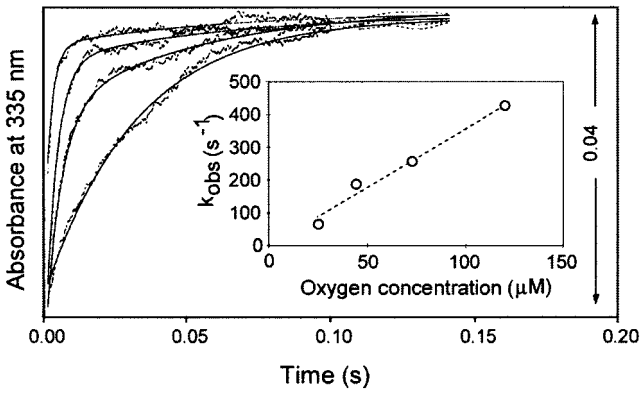

Figure 2. Time course of the changes generated following addition of oxygen to laccase incubated anaerobically with NO. Time courses followed at 330 nm in the stopped flow apparatus after mixing anaerobic laccase (65 μM) incubated with ~100 μM NO for 20 s with buffer at known oxygen concentrations. Oxygen concentration after mixing (bottom to top): 25, 45, 70, and 120 μM. The traces were fitted to a double-exponential increase. Insert: Plot of the observed pseudo-first-order rate constant of the fast component versus the oxygen concentration.

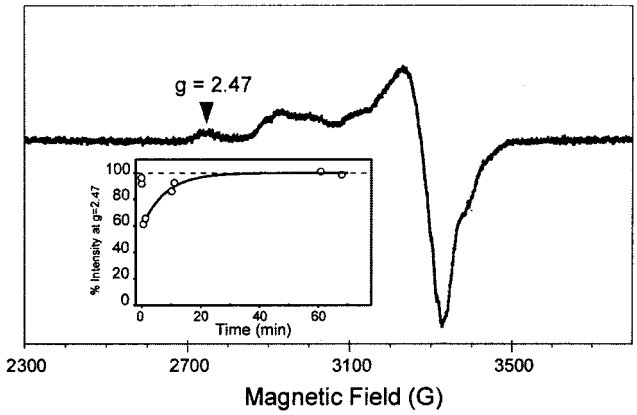

Figure 3. Time course of the events described in Figure 1 followed by EPR. EPR spectra of oxidized resting laccase at pH 6 (65 μM) measured at 77 K. Microwave power, 3.2 mW; microwave frequency, 9.4859 GHz; modulation frequency, 100 kHz; modulation amplitude, 1 G; time constant, 0.041 s; sweep rate, 9.15 G/s. The low-field component of the type 2 copper signal ($g = 2.47$) is indicated. Inset: Intensity of this component changing during the experiment and normalized with respect to the intensity obtained after 1 h. The first two points (before addition of 100 μM NO) were taken as a control, and the following measurements were taken at different time points (0.5, 1, 10, 11, 60, and 68 min) after the addition of NO. The line represents an exponential fit to the data.

niscent of the difference spectrum of a laccase intermediate.[20] This intermediate has been described as a peroxide, bridging oxidized T3 and reduced T2, and usually obtained by mixing oxygen with the three electron-reduced derivative T1Hg.[20] Addition of NO to anaerobic laccase generated only minor optical changes (Figure 1, spectrum C), but after addition of oxygen, spectrum A, was generated (Figure 1). Addition of oxygen to anaerobic laccase in the absence of NO did not produce any optical change. Similarly, addition of sodium nitrite (up to 5 mM) did not elicit the spectrum seen in Figure 1.

The amplitude of the change at 335 nm was the same at pH 7.4 or pH 6. At pH 6, the decay of this difference spectrum (Figure 1 and insert) was slow ($t_{1/2} \sim 15$ min). At pH 7.4 this decay rate fell ~5-fold ($t_{1/2} \sim 75$ min). Both the absolute rate of decay and its pH dependency are similar to those observed for the decay of the peroxide intermediate obtained using the T1Hg species.[20] Using the reported extinction coefficient for the intermediate, relative to the oxidized enzyme ($\Delta\epsilon_{335} \sim 5000$ M^{-1} cm^{-1}),[20] the amplitude of the decay at 335 nm (Figure 1, insert) is consistent with a concentration of ~18 μM for this species (i.e., 25% of the total laccase concentration used in the experiment). The final spectrum (Figure 1, spectrum B and insert), after the decay of the band at 335 nm, shows a remaining positive band at 330 nm which is consistent with the oxidation of ~25% reduced dinuclear T3 center ($\Delta\epsilon_{335} \sim 3600$ M^{-1} cm^{-1}; see above), present in the native enzyme. A similar difference spectrum was observed (not shown) after addition of H_2O_2 to laccase (H_2O_2/laccase molar ratio, 50:1), as reported previously.[21] Addition of NO to the sample incubated with H_2O_2 did not generate any optical change. Thus, the appearance of the features in spectrum A (Figure 1) are accompanied by changes consistent with oxidation of a fraction of T3. This is also expected for the formation of the peroxide intermediate.[20] Appearance of spectrum A (Figure 1) upon NO addition was also observed (not shown) when the enzyme had been previously incubated in 0.2 mM potassium ferricyanide for 1 h, in 1 mM fluoride for 30 min, or in 1 mM azide for 1 min.

The rate constant for the formation of the band at 335 nm in Figure 1 (spectrum A) was obtained by mixing oxygen containing buffer with an anaerobic solution containing the native enzyme plus NO (Figure 2). The time courses were fitted to a double-exponential curve.

The fastest of these processes was found to be oxygen concentration dependent, yielding a value of 3.5×10^6 M^{-1} s^{-1} for the second-order rate constant (Figure 2, insert), similar to that found for the formation of the peroxide intermediate in T1Hg.[15] The second process was slower ($k_{obs} \sim 30$ s^{-1}) and independent of the oxygen concentration. Use of sodium nitrite (5 mM) instead of NO did not elicit any changes.

Since the redox state of type 2 copper itself is difficult to detect optically, the changes occurring after addition of NO were monitored by EPR spectroscopy (Figure 3). This figure shows that the intensity of the low field component ($g = 2.47$) corresponding to the oxidized type 2 copper (see Materials and Methods) drops ~30−40% after the addition of NO (Figure 3, insert). This is followed by slow reoxidation. Note, however, that the first two measurements were obtained after 30 and 60 s after the addition of NO, and the percentage of T2 reduction could be even higher (see figure legend). This suggests that the reduction of the T2 coppers may not be restricted to the putative population of laccase having T3 reduced (23%). In fact, when the enzyme in the presence of oxygen was titrated with NO and the absorbance at 335 nm corresponding to the putative peroxide intermediate was followed optically, it was observed (Figure 4) that the maximum amplitude of the absorbance change was obtained when the molar ratio NO/laccase was 1:1. This indicates that a complete formation of the putative peroxide intermediate is only obtained at equimolar concentrations of NO and laccase. Since the formation of the peroxide intermediate requires the reduction of T2, we conclude therefore that the affinity of T2 for NO may be similar, irrespective of the redox state of T3.

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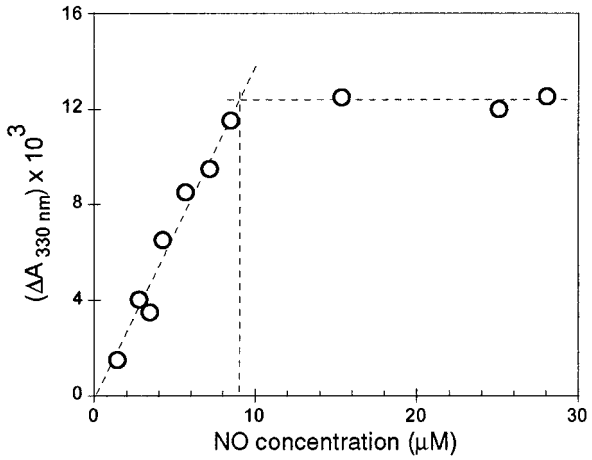

Figure 4. Effect of substoichiometric additions of NO to laccase in the presence of oxygen. Titration corresponding to the changes at 330 nm after the addition of alliquots of NO to 8.5 μM laccase in the presence of oxygen. The time interval between NO addition and measurement was less than 5 s. The vertical dotted line indicates the enzyme concentration.

Discussion

On addition of NO to an aerobic solution of laccase or addition of oxygen to anaerobic solutions of laccase in the presence of NO, rapid absorbance changes occur, as depicted in Figures 1 and 2. The question arises as to whether these changes are due to the direct reaction of NO and O_2 with laccase or indirectly to the reaction of the products of the reaction between NO and O_2 with the enzyme.

In aerobic aqueous solutions nitrite is essentially the only product of the reaction between NO and O_2.[22] Not only does addition of nitrite to oxidized laccase not generate the spectra observed, but the formation of nitrite is in any case much too slow to account for our observations.[23] Although higher nitrogen oxides are formed as intermediates in the reaction of NO and O_2, the calculated concentrations[22] are far too small to be responsible for the observed stoichiometric changes in the laccase copper centers. The concentrations of N_2O_3 and NO_2 may be calculated to be 1 and 0.4 nM, respectively, versus a laccase concentration of 65 μM. We conclude therefore that the spectra we have described are due directly to the action of NO and not to the products of its reaction with O_2.

In fact, the changes observed after addition of NO to laccase in the presence of oxygen can be interpreted as due to the formation of the peroxide intermediate in a fraction (25%) of the molecules. However, to achieve this, a fully reduced trinuclear center (T3 and T2) must be present,[6] which upon reaction with oxygen would produce the peroxide intermediate. Whereas we observe reduction of T2 by NO (see Figure 2), it seems unlikely that NO also reduces T3. First, because reduction of T3 by NO has been reported to be very slow,[16] and second, because of the 1:1 molar stoichiometry between NO and laccase observed in the appearance of the band at 335 nm (Figure 4). The obvious alternative is that a fraction of T3 is already reduced before addition of NO, as suggested previously.[21] Indeed, our data are consistent with 25% of the molecules of the native enzyme containing reduced T3 that become oxidized upon NO addition and oxygen binding. Similar spectral changes occurred after addition of hydrogen peroxide, an oxidant for T3 copper, consistent with oxidation of 25% of T3, also reinforcing this hypothesis. Furthermore, addition of NO to this fully oxidized sample did not give rise to optical changes. Also, upon mixing oxygen with the enzyme anaerobically incubated with NO, the band at 335 nm is formed with a second-order rate constant of $3.5 \times 10^6\ M^{-1}\ s^{-1}$. This value is identical, within experimental error, to $2 \times 10^6\ M^{-1}\ s^{-1}$, reported for the formation of the peroxide intermediate when mixing oxygen with fully reduced T1Hg,[15] and also similar to $5 \times 10^6\ M^{-1}\ s^{-1}$, obtained for the formation of the native intermediate when mixing oxygen with the fully reduced enzyme.[22] In the last case, although the formation of the native intermediate is associated with oxidation of T1, the same kinetic process should be observed (e.g., oxidation of T3 coppers), as oxidation of T3 is the rate-limiting step.[20]

The fact that the spectral changes attributed to the peroxide intermediate are observed upon NO addition, even after preincubation of laccase with ferricyanide or fluoride, is consistent with the fact that ferricyanide cannot oxidize the dinuclear center T3.[21] Therefore, the population of molecules containing reduced T3 is still able to form the peroxide intermediate. Fluoride, on the other hand, is a strong ligand ($K \sim 10^4\ M^{-1}$)[24] of oxidized type 2 copper, which might preclude the formation of the intermediate. Fluoride, however, does not bind T2 when the dinuclear center T3 is reduced.[24] The observation that the intermediate is formed also after incubation of laccase with azide is intriguing, as azide has been reported to bind preferentially to T2 when T3 is reduced.[7] The reaction may still be possible, however, if NO can reduce T2 and the peroxide group bridges between T2 and the T3 copper that is not bound to azide. This reaction possibly displays different kinetics.

The fact that the change in amplitude at 335 nm is complete at a stoichiometry of 1:1 NO/laccase suggests that all the T2 sites are reduced at the end of the titration, or at least bind NO. Thus, although the affinity of NO for T2 copper may be different depending on the redox state of T3, as for azide[7] or fluoride,[24] both fractions of the enzyme bind NO with an affinity $> 10^6\ M^{-1}$. Furthermore, the fact that other centers (T3 or T1) did not become reduced suggests that only one NO is bound per molecule, at T2. The absence of bleaching of the bands at 614 or 330 nm after addition of NO to the anaerobic enzyme provides further evidence that binding of NO and reduction of Cu must occur at the optically undetectable T2 copper, confirming previous results[16] which describe this center as the electron entry site in the reduction of laccase by NO. Furthermore, although the EPR results (Figure 3) suggest that only a fraction (30−40%) of the T2 copper becomes reduced, it is possible that low-temperature effects leading to electron redistribution[16,25] between T2 copper and NO may mask the true percentage of reduced T2 copper at room temperature. Formation of the native

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(23) For example in Figure 2 an anaerobic solution of 65 μM laccase/100 μM NO was mixed with 25 μM oxygen and the spectrum described in Figure 1 appeared at a rate of approximately 50 s^{-1}. The rate equation for the reaction of NO with O_2 may be written as $d[NO_2^-]/dt = 4k_1[NO]^2[O_2]$, where $k_1 = 2 \times 10^6\ M^{-2}\ s^{-1}$. Given the second-order nature of this process in [NO], the chemical oxidation of NO is far too slow to produce species relevant to the laccase reactions observed here. The calculated initial rate for nitrite formation in the above case is $2 \times 10^{-6}\ M\ s^{-1}$, which indicates that under the concentration regime employed the half-time for the reaction is >40 s, whereas the reaction with the laccase has a half-time of some 14 ms.
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intermediate, as a product of the decay of the peroxide intermediate, was not detected, either by optical means or by EPR. This intermediate, which displays a band at 360 nm relative to the oxidized enzyme,[22] is formed upon electron transfer from T1 to the peroxide intermediate and has been associated with an oxygen radical which is detected only at low temperature.[26] Although such a species may be formed in our sample as the product of the decay of the peroxide intermediate, the fact that in our system the peroxide intermediate must be reduced by T2 ($t_{1/2}$ of minutes), and not by T1 ($>1000\ s^{-1}$), suggests that its formation is probably paralleled by its decay, and a significant population never accumulates.

In summary, we have observed rapid reduction of a type 2 copper in laccase upon NO addition. This has been observed directly by EPR and also by optical means through a subpopulation of the enzyme (~25%) which contains a dinuclear T3 center already fully reduced (2 electrons). The kinetics of the formation of the peroxide intermediate are virtually identical to those reported for the formation of the native intermediate using fully reduced enzyme, or the formation of the peroxide intermediate using fully reduced T1Hg. The rate of decay of the species described is very slow and similar to the decay of the peroxide intermediate, showing also a similar pH dependency. We conclude that the peroxide intermediate has been formed via reduction of T2 copper by NO in a fast reaction. We suggest that this reaction may not only hold implications for the regulation, by NO, of enzymes containing trinuclear copper centers (e.g. the major copper protein in blood plasma, ceruloplasmin), but also could be relevant to the regulation of copper enzymes in general. In addition, the method we report here for the formation of the peroxide intermediate in laccase molecules which possess the full complement of copper atoms should prove useful to the study of the catalytic cycle of this enzyme. For example, it permits one to investigate the O−O scission step by the addition of a single electron through the native T1 copper. This reaction cannot be so easily isolated by other methods.

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