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Visible Light-Driven O_2 Reduction by a Porphyrin–Laccase System

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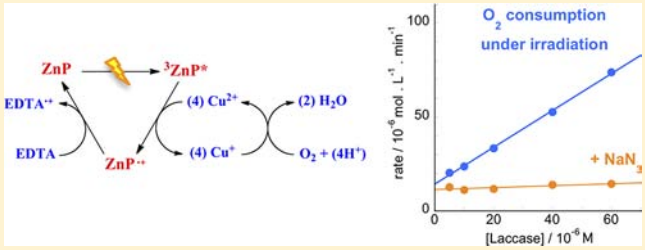
Supporting Information

ABSTRACT: Several recent studies have shown that the combination of photosensitizers with metalloenzymes can support a light-driven multielectron reduction of molecules such as CO_2 or HCN. Here we show that the association of the zinc tetramethylpyridinium porphyrin ($ZnTMPyP^{4+}$) photosensitizer with the multicopper oxidase (MCO) laccase allows to link the oxidation of an organic molecule to the four electrons reduction of dioxygen into water. The enzyme is photoreduced within minutes with porphyrin/enzyme ratio as low as 1:40. With a 1:1 ratio, the dioxygen consumption rate is 1.7 μmol L^{-1} s^{-1}. Flash photolysis experiments support the formation of the triplet excited state of $ZnTMPyP^{4+}$ which reduces the enzyme to form a radical cation of the porphyrin with a $k_{ET} \approx 10^7$ s^{-1} M^{-1}. The long-lived triplet excited state of the $ZnTMPyP^{4+}$ (τ_0 = 0.72 ms) accounts for a substantial electron-transfer quantum yield, ϕ_{ET} = 0.35. Consequently, the enzyme-dependent photo-oxidation of the electron donor occurs with a turnover of 8 min^{-1} for the one-electron oxidation process, thereby supporting the suitability of such enzyme/sensitizer hybrid systems for aerobic photodriven transformations on substrates. This study is the first example of a phorphyrin-sensitized four-electron reduction of an enzyme of the MCO family, leading to photoreduction of dioxygen into water.



INTRODUCTION

An effective utilization of solar energy to drive catalysis requires the development of robust systems where light absorption triggers electron transfer (ET) events that subsequently lead to the activation of a catalytic center.[1] Examples of association of photosensitizers with transition metal complexes successfully used for photocatalysis have been recently flourishing in the literature.[2] Among other reactions, oxygenations have been described to proceed with water as the sole source of oxygen atom through the photogeneration of metal-oxo (M═O) species due to an ET to a sacrificial electron acceptor via a ruthenium polypiridyl complex.[2di] Although allowing the oxygenation of sulfur- or alkene-containing substrates in substantial yields, such photocatalytic systems are intrinsically limited by the requirement of a sacrificial acceptor of electrons. This requirement, in addition to the need to maintain an overpressure of an inert gas, considerably reduces the sustainability of the process. Therefore, open-to-air systems that could couple a catalyst to a renewable electron acceptor source (i.e., O_2) are highly desirable. One direction of research lies in the design of hybrid catalysts associating a photosensitizer with a unit capable to efficiently react with dioxygen at a substantial turnover rate. Among possible candidates for this "oxygen catalytic units" are several metallo-enzymes. Photoactivation of metallo-enzymes is a growing field in which Gray, Winkler, and co-workers pioneered the coupling of photoactive units to metallo-enzymes.[3]

In nature, the four-electron redox reactions of the O_2/H_2O couple are carried out by a discrete number of metallo-proteins that are either membrane embedded complexes, i.e., the Mn_4CaO_x oxygen evolving complex (OEC) of the plant photosystem II (PSII) or the heme aa_3-Cu_B cytochrome *c* oxidase (C*c*O) of respiratory chains, or soluble oxidases. Multicopper oxidases (MCOs) contain a unique set of copper ions made of at least one each of the three types of biorelevant copper sites: mononuclear type 1 (T1) and type 2 (T2), and binuclear type 3 (T3). They couple dioxygen reduction to the oxidation of substrates, either organic or metal ions.[4] Typical of MCOs, the enzyme laccase[5] (EC1.10.3.2, from plant, fungi, or bacteria) is a robust catalyst largely studied for its potential uses in industrial processes,[6] in organic synthesis applications[7] and as cathodic catalyst reducing dioxygen into water in biofuel cells.[8]

The laccase scaffold consists of a triplicated domain homologous to the blue copper proteins cupredoxins (e.g., azurin, plastocyanin, rusticyanin). Evolving from a single domain ET protein to a three domains oxidase (domains D1,

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D2, D3), proto-laccase lost blue copper sites (T1) in D1 and D2, acquired a T2-T3 trinuclear copper site mapping at the boundaries of D1 and D3 and substrates binding sites in neo-formed clefts[9] (Figure 1). The surface located substrate

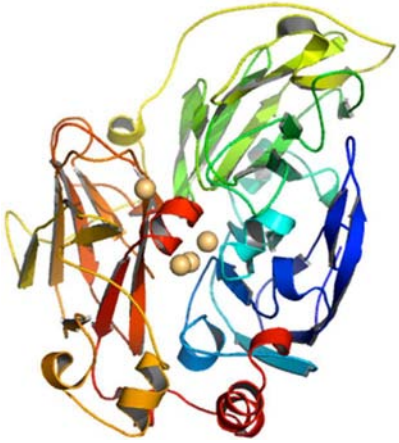

Figure 1. Three-dimensional representation of a fungal laccase based on the coordinates of the PDB entry 2HRG. Cupredoxin domains are colored from blue (D1) to green (D2) to red (D3). Light-orange spheres depict copper atoms, an isolated Cu(II) T1 close to the surface of the enzyme in D3 and a Cu(II) T2-T3 trinuclear cluster embedded in the enzyme at the D1-D3 boundaries.

oxidation center (in D3) holds the blue copper-binding site (or T1) in which the copper ion is coordinated by the side chains of two histidines and one cysteine. This site exhibits an intense Cys(S)→Cu(II) charge-transfer (CT) band in laccase absorption spectrum ($\varepsilon \approx 5000-6000$ M^{-1} cm^{-1} at about 600 nm) resulting in the intense blue color of the oxidized enzyme and a very small parallel hyperfine coupling constant ($A_{\|}$) in its electron spin resonance (ESR) spectrum.[10] The dioxygen reduction center consists of a trinuclear copper cluster (TNC) structured around a pair of antiferromagnetically coupled copper ions coordinated to histidines (T3 copper site) and a two histidines/H_2O coordinated copper ion (T2 copper site). The oxidized T3 copper site (similar to that present in catechol oxidase and tyrosinase) is characterized by a bridging hydroxide to Cu(II) CT transition at 330 nm ($\varepsilon \approx 5000$ M^{-1} cm^{-1}). The oxidized T2 copper site exhibits spectral features reminiscent of tetragonal cupric complexes (i.e., no detectable transition in the UV/vis region and a large $A_{\|}$ coupling constant in its ESR spectrum).[10] Substrate oxidation and oxygen reduction sites are located ~13 Å away from each other and are connected through a histidine-cysteine-histidine tripeptide (H−C−H) involved in the coordination of the metal ions. Laccases do not have a well-defined binding pocket for substrate but rather a shallow depression at the bottom of which the distal histidine coordinating the T1 copper ion points out. Substrate oxidation is strictly outer-sphere and involves a nearby acidic residue for proton abstraction. The bimolecular reaction is fast ($\sim 10^5-10^6$ s^{-1} M^{-1}), and ET rates can be even faster with non-deprotonable substrates. Subsequently, one electron is transferred from the reduced T1 copper ion (Cu(I)) to the oxidized TNC (intramolecular ET) where eventually the four electrons reduction of O_2 takes place through two sequential two-electron reduction steps: dioxygen reaction with a reduced TNC to generate a Cu(II)−(O_2)−Cu(II) peroxide species preceding a reductive cleavage of the O−O bond that triggers movements of protonated oxygen atoms away from the cluster.[11]

Laccases can be reduced by photogenerated radicals like CO_2^{-}[12] or lumiflavin semiquinone.[13] Laccase-catalyzed oxidations of transition metal complexes have been also reported illustrating the ability of the enzyme to interact with such partners.[14] Recently, we described the first example of photodriven dioxygen reduction by a laccase.[15] We demonstrated that, in the presence of an exogenous electron donor (EDTA) and ruthenium polypyridine-type photosensitizers, the enzyme can be fully reduced and is in turn able to reduce dioxygen. Beyond the demonstration of the photodriven reduction of dioxygen, the availability of various photosensitizers and sacrificial electron donors/acceptors as well as the plasticity of laccases encourage studies toward such new hybrid materials with potentials applications in photocatalysis, photonics, and energy. There are multiple directions of research to understand better the new hybrids as well as to improve their performance. To list a few, those include the utilization of sensitizers with different photophysical properties, the organization of the interaction (e.g., through co-adsorption,[16] a covalent bond,[17] or ion-pair cofacial complex formation[18]), or developments toward the use of less expensive materials.

Porphyrins are well-studied redox-active photosensitizers most of which display remarkable long lifetime of the triplet excited state.[19] They offer the possibility to replace ruthenium complexes by a photosensitizer which either contains an abundant first row metal, or is even a metal-free photosensitizer. One challenging issue in the design of man-made mimicks of photosynthesis is the efficient accumulation of multiple charges at the catalytic unit. Only few recent examples of multielectron or hole accumulations have been reported, some of which utilize metalloproteins.[15−17] This paper describes a laccase−porphyrin system which is catalytically active for dioxygen reduction—the first example of laccase powered by porphyrin as a photosensitizer and only the second example altogether of a photocatalytic action of laccase, following our first study which employed ruthenium polypyridyl systems as photosensitizers. In the presence of an excess of the enzyme, transient absorption data suggest a formation of the long-lived radical cation of $ZnTMPyP^{4+}$ from the triplet excited state of the zinc porphyrin, ^{3}ZnP*. The long, hundreds of microseconds, lifetime of the ^{3}ZnP* state accounts for a relatively high quantum yield for the reduction of the enzyme, and for the overall high efficiency of dioxygen reduction by the porphyrin−laccase system.

EXPERIMENTAL SECTION

Laccase Production. The recombinant laccase (LAC3 from *Trametes* sp. C30)[20] was purified from the broth of a transgenic *Aspergillus niger* grown in a 10 L fermentor (BioFlow 3000, New-Brunswick) as described elsewhere.[21]

Synthesis of the Porphyrin. 5,10,15,20-Tetrakis(4-*N*-methylpyridinium)porphyrinatozinc chloride, $[ZnTMPyP]Cl_4$, was synthesized using a modified literature process.[22] One gram (1.5 mmol) of 5,10,15,20-tetrakis(4-pyridinium)porphyrinatozinc, [ZnTPyP],[23] was suspended in 15 mL of dimethylformamide, and 4 mL of methyl iodide was added. The mixture was stoppered and stirred for 5 days at room temperature. After completion of the reaction, the resulting solution was poured into 100 mL of diethyl ether, and the precipitated solid was collected by filtration, washed with diethyl ether (3 × 10 mL), and dried under vacuum to give $[ZnTMPyP]I_4$ as a dark purple solid (yield 1.69 g, 80%). The iodide was converted to the chloride by passing a solution of $[ZnTMPyP]I_4$

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in the minimum amount of water through a Dowex 2 anion-exchange column in the Cl^- form.

Kinetic Study of Bleaching of the Laccase T1 Cu(II) Absorption. Decrease of the absorption at λ = 605 nm as a function of the irradiation time was monitored using a VARIAN Cary 50 spectrophotometer equipped with a homemade 90° irradiation setup. Experiments were performed in Britton Robinson (B&R) buffer pH 4.0 with 0.025:1 $ZnTMPyP^{4+}$:laccase (40 μM) ratio and 100 equiv of EDTA as a sacrificial electron donor. Samples were placed under a continuous flow of argon in rubber-jacketed cuvettes. Irradiations were performed at room temperature using a Dolan-Jenner MI-I50 illuminator (EDMUND) equipped with a 10 mm light conducting fiber. ESR spectra were obtained using a Bruker Elexsys E500-9.5/2.7 spectrometer equipped with a BVT 3000 digital temperature controller (100−400 K). The experiments were performed using different laccase:$ZnTMPyP^{4+}$ ratios ranging from 5:1 to 1:1 and 100 equiv of EDTA as sacrificial electron donor. Samples (320−450 μM laccase, 0.2−1 equiv of $ZnTMPyP^{4+}$, and 100 equiv of EDTA in B&R buffer set at pH 4.0) were placed in Young valved ESR tubes in the absence of dioxygen. In some cases, a long pass 410 nm filter was placed between the light source and the samples in order to cut-off UV radiation. Irradiation times were up to 300 s. The samples were analyzed in the spectrometer at 115−120 K as frozen solutions.

Stopped-Flow. Stopped flow measurements were performed on a Bio-Logic SFM 300 system (Bio-Logic, Grenoble, France) equipped with a MOS-200M spectrometer comprising a Xe/Hg 150-W lamp, a PMT-250 photomultiplier, and a motorized monochromator. Argon-flushed syringes were filled with thoroughly degassed solutions: S1 = 20−80 μM laccase in B&R buffer pH 4.0; S2 = 60 μM coniferyl alcohol in B&R buffer pH 4.0. Delay lines, the mixing chamber, and the Z-shaped microcuvette (1 cm light path) were extensively rinsed with degassed B&R buffer pH 4.0 prior to shots. Experiments were performed at 20 °C in B&R buffer set at pH 4.0, and the reaction was initiated by mixing a solution of laccase with a solution of substrate. The amount of oxidized T1 copper center was followed by monitoring changes in its absorption at 605 nm.

Dioxygen Consumption. Dioxygen consumption was measured by polarography using a model 781 oxygen meter (Strathkelvin Instruments) with a Clark microelectrode fitted to a temperature-controlled glass chamber (1.5 mL). Irradiation of the sample (typically 40 μM laccase, 0.1 equiv of $ZnTMPyP^{4+}$, and 100 equiv of EDTA) was performed through the glass chamber using a Dolan-Jenner MI-150 illuminator (Edmund) equipped with a 150-W EKE quartz halogen lamp with optic fibers (diameter 0.8 cm) adapted to the respiratory chamber. A power density of about 230 mW/cm^2 was measured with a power meter Vector H410 connected to a Scientech head after a 2 cm thick water lens corresponding to the water cooled glass respiratory chamber.

Flash and Steady-State Photolysis. Flash photolysis experiments were performed on a home-built setup briefly described previously[24] upgraded with a tunable excitation source. The samples were selectively excited at 438 nm (sample of $ZnTMPyP^{4+}$ porphyrin on its own) or 439 nm (for porphyrin/laccase sample) into the porphyrin Soret band with the frequency-doubled output of a homemade Ti:sapphire tunable nanosecond pulsed laser (10 Hz repetition rate, 25 ns pulse width). The energy of the excitation pulses at the sample was ~1.5 mJ. The Ti:sapphire laser was pumped with the second harmonic (532 nm) of a Q-switched Nd:YAG laser LS-2137U (LOTIS TII). A 150-W Xe arc lamp (Hamamatsu, Japan) was used as the probe light source. The probe light was detected through a SPEX MiniMate monochromator by a custom-built detector unit, based on a FEU-118 PMT. The detector current output was coupled into a Tektronix TDS 3032B digital oscilloscope and subsequently transferred to the computer. The same tunable Ti:sapphire nanosecond pulsed laser was used as the excitation source for steady-state photolysis experiments. The laser output was set at 439 nm to excite selectively into the porphyrin Soret band with the average irradiation power of 16 mW (10 Hz repetition rate per 1.6 mJ pulse energy).

The solutions for the flash photolysis experiments contained 5 × 10^{-6} M $ZnTMPyP^{4+}$ and 75 × 10^{-6} M laccase in B&R buffer at pH 4.0. For steady-state photolysis experiments, solutions contained 5 × 10^{-6} M $ZnTMPyP^{4+}$, 75 × 10^{-6} M laccase, and 0.57 × 10^{-3} M EDTA in B&R buffer at pH 4.0. The porphyrin concentration was controlled spectrophotometrically based on the extinction coefficient ε = 2.04 × 10^5 M^{-1} cm^{-1} for the free $ZnTMPyP^{4+}$ at the Soret peak at 437 nm.[25]

All flash photolysis and steady-state photolysis experiments were performed in quartz cells with 1 cm path-length, on deoxygenated samples, degassed by the freeze−pump−thaw technique unless noted otherwise.

The analysis of time-resolved data to obtain decay lifetimes was performed using Igor Pro software (WaveMetrics, Inc.). The decay traces were fitted to the (multi)exponential decay law using least-squares algorithm built into Igor Pro. Global fitting was applied to analyze simultaneously decay kinetics obtained for numerous spectral points, which enabled to reconstruct the shape of transient spectra and produced reliable values of the lifetimes (see Supporting Information).

RESULTS

Photoreduction of the Enzyme. The anaerobic photoreduction of laccase was monitored in the presence of EDTA as sacrificial electron donor and with or without $ZnTMPyP^{4+}$. Kinetics of bleaching of the λ_{605} absorption band of deoxygenated samples upon irradiation are presented in Figure 2. In the absence of $ZnTMPyP^{4+}$, the T1 Cu(II) ion of laccase

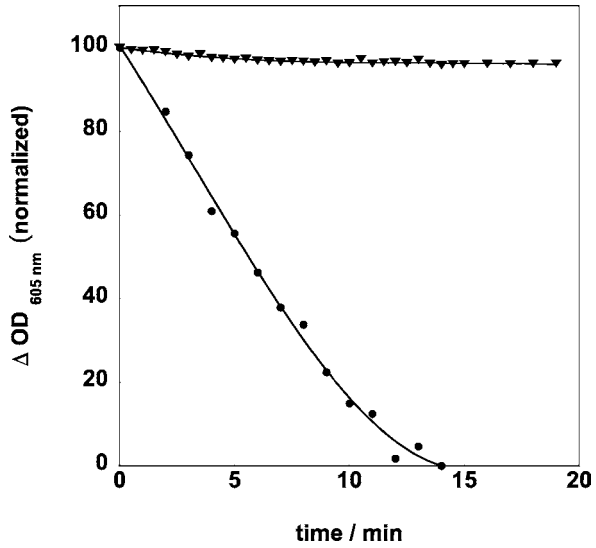

Figure 2. Kinetics of bleaching of the laccase T1 Cu(II) absorption band. Argon-flushed B&R buffer solution at pH 4.0 containing 40 μM laccase and 4 mM EDTA in the absence (▼) or presence of 1 μM $ZnTMPyP^{4+}$ (●). OD variations are expressed as % of the initial absorption of each sample. Fitting curves (polynomial) are represented as black solid lines. Irradiation performed with a Dolan-Jenner MI-150 illuminator equipped with a 150-W EKE quartz halogen lamp with an optic fiber (power density 235 mW/cm^2).

was barely photoreduced within the course of the experiment whereas in the presence of $ZnTMPyP^{4+}$, a substantial bleaching of the T1 Cu(II) ion absorption was achieved within minutes, suggesting a promoting role of the porphyrin in the photoinduced ET to the enzyme.

In order to visualize an ET to the TNC, ESR measurements were performed. Deoxygenated samples of laccase were irradiated in the presence of EDTA and variable concentrations of $ZnTMPyP^{4+}$ in B&R buffer at pH4 in ESR quartz tubes. Before irradiation, the 120 K spectrum displays the characteristic signals of the Cu(II) ions of the enzyme (Figure 3): the T1 Cu(II) ion signal can be modeled with the parameters $g_{\|}$ =

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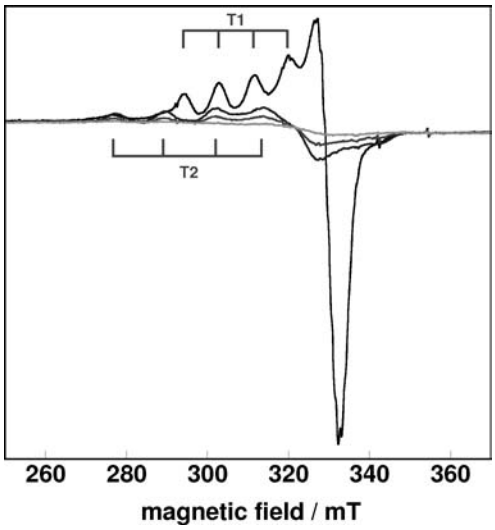

Figure 3. Evolution of the X-band ESR spectrum of 490 μM laccase in the presence of 1 equiv of $ZnTMPyP^{4+}$ and 50 equiv of EDTA under white light irradiation at pH 4.0 under inert atmosphere. X-band ESR spectra were recorded in frozen solutions at 120 K before irradiation (black line) and after 30, 120, and 300 s of irradiation (gray gradient for the corresponding lines). Microwave power 20 mW, modulation 3 G, gain 10^5.

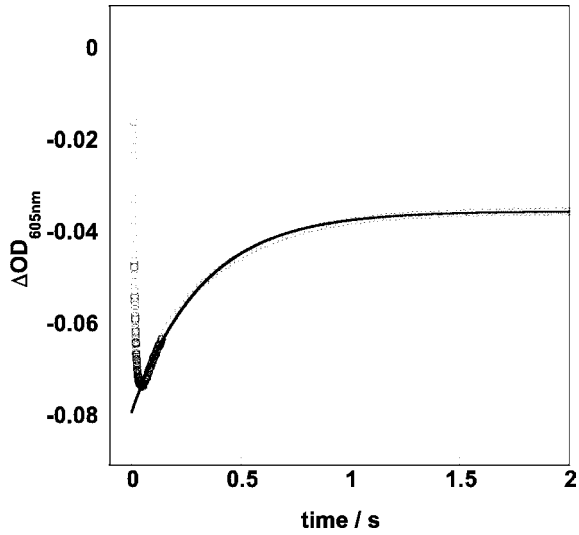

Figure 4. Transient-state kinetics obtained after mixing equal volumes of oxidized laccase with coniferyl alcohol at final concentrations of 30 μM in degassed B&R buffer pH 4.0 at 20 °C. Absorption changes monitored at 605 nm; solid line, single-exponential fit (k = 3.1 s^{-1}).

2.19, $A_{\parallel}$ = 87 G, and $g_{\perp}$ = 2.04, and the T2 Cu(II) with the parameters $g_{\parallel}$ = 2.25 and $A_{\parallel}$ = 162 G. These parameters are similar to those previously reported for LAC3 laccase and other laccases.[20,26] After 30 s of irradiation, the blue color disappeared as well as the ESR signal arising from the T1 Cu(II) indicating that at least one electron was transferred to the enzyme. In the presence of substoichiometric amounts of $ZnTMPyP^{4+}$, a reduction of the T2 Cu(II) required long additional irradiation times and the reduction was not complete even after 30 min of irradiation. The complete reduction of the T2 copper was achieved within ~4 min when the $ZnTMPyP^{4+}$ and the laccase were used in equimolar amount (see Figure 3, light gray line). The reduction of the enzyme with $ZnTMPyP^{4+}$ occurs much faster than that we previously observed with ruthenium complexes.[15] This experiment indicates that a minimum of two electrons were transferred from the photosensitizer to the enzyme under irradiation. Upon reoxygenation, both the T1 and T2 coppers were reoxidized as assessed by the reappearance of the blue color and of the ESR features as previously observed.[15]

Stopped-Flow. Intramolecular ET in laccase can be monitored with a stopped-flow system following spectral changes in the UV/vis region. When a solution of laccase was mixed with equimolar quantities of a substrate (i.e., coniferyl alcohol), the T1 Cu(II) ion was reduced in a fast bimolecular process as attested by the decrease of the absorbance at 605 nm (Figure 4). The reduction process follows a second-order rate law with an intermolecular ET rate constant of 8.3×10^5 M^{-1} s^{-1}. This reduction phase is followed by a slow reoxidation process as attested by a reappearance of the transition at 605 nm. Experiments were reproduced using concentrations of laccase ranging from 10 to 40 μM. The reoxidation process was found independent of protein concentration and was assigned to an intramolecular ET from the T1 Cu(I) to T3 Cu(II). The slow reoxidation phase can be fitted with a single-exponential function corresponding to a rate constant of 3.1 s^{-1} (see Figure 4). From the analysis of the amplitude of the absorption changes at 605 nm it appeared that the T1 Cu(II) ion is only partially reoxidized and an equilibrium constant K = 0.8 at 20 °C was calculated for the couple T1Cu(I)/T3Cu(II). Using this value of K and eq 1, a difference between the redox potential of the T1 and T3 centers of 6 mV was calculated. A redox potential of 680 mV has been previously measured for the T1 copper of this enzyme;[14a] therefore, one can calculate a redox potential of 674 mV for the T3 center.

$$E^0_{T1} - E^0_{T3} = -0.059 \log(K) \quad (1)$$

Dioxygen Consumption Rates. As previously demonstrated,[15] a photoreduced laccase converts O_2 into water. Using a Clark electrode, we measured the consumption of dioxygen at 25 °C in an air-saturated buffered sample set at pH 4.0. In the dark, in the absence of photosensitizer or EDTA or when $ZnTMPyP^{4+}$ was used alone, no significant dioxygen consumption was detected. Upon irradiation of solutions containing $ZnTMPyP^{4+}$ + EDTA with or without laccase (0–60 μM), the dioxygen concentration decreased proportionally to the concentration of enzyme (Figure SI1A). Light-induced dioxygen consumption rates obtained with variable concentrations of $ZnTMPyP^{4+}$ ranged from 3 to 100×10^{-6} mol L^{-1} min^{-1} upon addition of laccase (Figure SI1B). The enzyme-dependent dioxygen consumption was inhibited in the presence of 10 mM NaN_3 (Figure SI1). At this concentration of azide the enzyme is totally inhibited.[15] The residual consumption, i.e., the enzyme-independent consumption, represents less than 25% of the maximum rate (2 to 20×10^{-6} mol L^{-1} min^{-1}). The maximum enzyme activity observed in the presence of $ZnTMPyP^{4+}$ is more than 1 order of magnitude higher than that we previously observed in the presence of $Ru(bpy)_3^{2+}$ under similar conditions (bpy = 2,2′-bipyridine).[15]

Steady-State Photolysis. The absorption spectrum of the $ZnTMPyP^{4+}$/laccase mixture (1:15 mol:mol, B&R buffer at pH 4.0) is dominated by the broad absorption of the enzyme's T1 copper site positioned at ~600 nm along with the intense Soret absorption band of the porphyrin at 439 nm while a feature due to the porphyrin Q (0,1) absorption band is seen at ~568 nm

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(Figure SI2). The most intense Soret absorption band of $ZnTMPyP^{4+}$ (B&R buffer at pH 4) shifted slightly, from 437 to 439 nm, upon addition of 15 equiv of laccase.

Steady-state photolysis experiments for $ZnTMPyP^{4+}$ in the presence of 15 equiv of laccase were performed in anaerobic conditions in order to accumulate a detectable quantity of reduced laccase. The experiment was conducted in presence of 100 equiv of sacrificial electron donor EDTA to replenish the initial form of the porphyrin $ZnTMPyP^{4+}$ after its oxidation in the process of ET to laccase. To rule out any potential effect of the direct excitation of laccase, the steady-state photolysis was performed using selective laser excitation into the porphyrin Soret band at 439 nm. Selective photoexcitation of the porphyrin leads to clear evidence of the reduction of the laccase, manifested by disappearance of intrinsic absorption band of the T1 copper site in laccase in the 600 nm region (Figure 5). The porphyrin Soret band was also bleached after

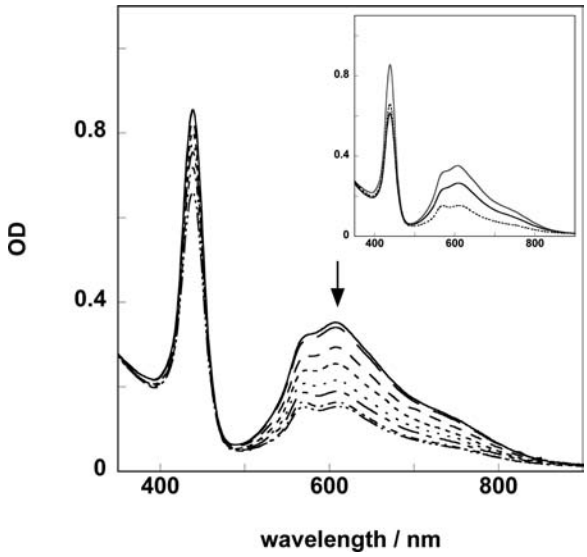

Figure 5. Absorption spectral changes in the course of steady-state photolysis of $ZnTMPyP^{4+}$ with laccase in the presence of EDTA, in B&R buffer at pH 4.0. Irradiations with 439 nm laser light, 16 mW average power, 1 cm path length, deoxygenated sample (0–2560 s total irradiation time). Inset: changes in the absorption spectra illustrating a partial recovery of laccase after photolysis. Solid gray line, absorption spectrum of deoxygenated sample before photolysis; dashed line, spectrum after 2560 s total irradiation of the deoxygenated sample; solid black line, spectrum of the sample after the photolysis followed by bubbling the sample with air.

prolonged irradiation (~45 min); however, the change of porphyrin absorption was less pronounced than that of laccase (23% for ZnP and 57% for laccase).

The photoreduction of laccase was mostly reversible, as is illustrated by the inset of Figure 5. When the photolyzed solution was bubbled with air, the T1 absorption of laccase was restored to ~75% of the initial pre-photolysis value in the time scale of the experiment due to the reduced laccase being reoxidized by oxygen to the initial state. However the bleaching of the porphyrin Soret band was irreversible, which could be due to the porphyrin being directly reduced by EDTA in a photoinduced reaction with no involvement of laccase, as previously documented.[27,28]

Flash Photolysis. Flash photolysis studies were performed under 438 nm (sample of $ZnTMPyP^{4+}$ porphyrin on its own) or 439 nm (for porphyrin/laccase sample), 25 ns laser pulse excitation in the range 380–770 nm. Kinetic traces were recorded with a 10-nm step, and the transient spectra reconstructed using a global fit analysis (see SI). Representative traces are shown on Figure 6. The transient spectrum of

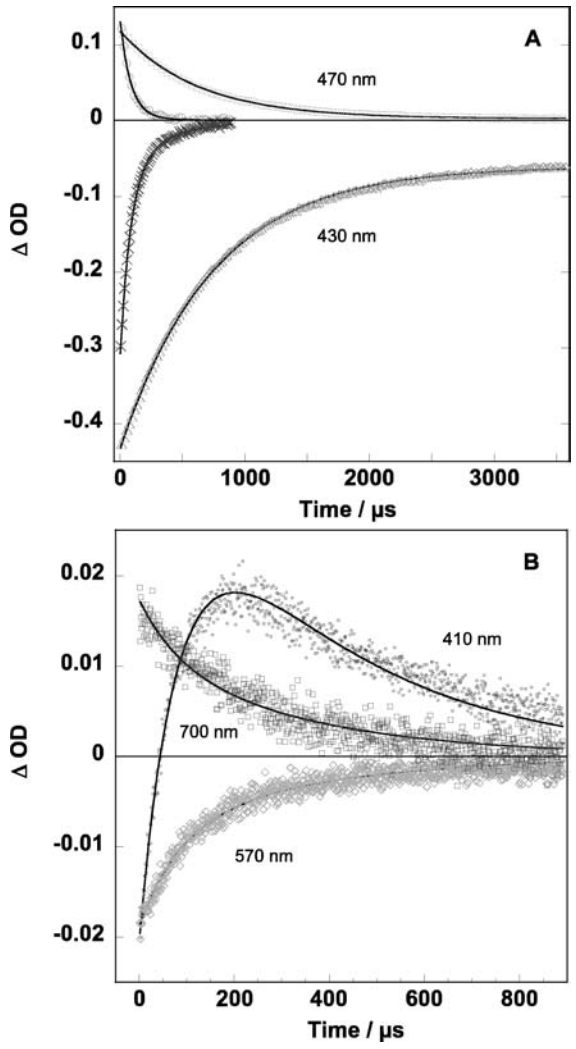

Figure 6. (A) Representative transient absorption decay traces obtained for $ZnTMPyP^{4+}$ with laccase (○, ×) and without laccase (□, △) in B&R buffer at pH 4.0 following excitation with 438/439 nm, ~25 ns laser pulse. For the $ZnTMPyP^{4+}$ without laccase, solid lines correspond to monoexponential fit to the data with the lifetime of 0.72 ± 0.07 ms. (B) Representative transient absorption decay traces obtained for $ZnTMPyP^{4+}$ with laccase. Solid lines correspond to the biexponential global fit to the data with the lifetimes of τ_1 = 84 ± 8 μs and τ_2 = 350 ± 10 μs.

$ZnTMPyP^{4+}$ without laccase agrees well with the known spectrum of the triplet excited state of the porphyrin[29] and decays uniformly with a lifetime of 0.72 ± 0.07 ms. In the presence of laccase, the transient spectrum obtained immediately after excitation (Figure 7, ▼) also agrees well with the known triplet–triplet absorption spectrum of Zn porphyrin[29,30] and indicates the population of the lowest triplet excited state of $ZnTMPyP^{4+}$ by the end of the laser pulse. Differently to the case of the porphyrin-only flash photolysis, this spectrum evolves on a time scale of tens of microsecond into that of

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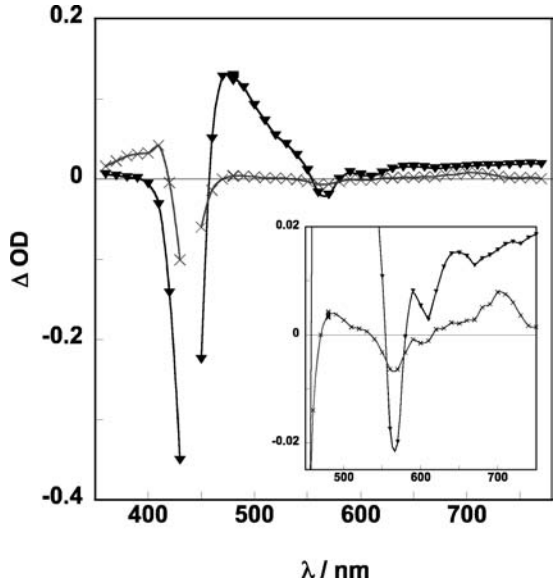

Figure 7. Transient absorption spectra obtained in laser flash photolysis for $ZnTMPyP^{4+}$ with laccase, B&R buffer pH 4.0, excitation at 439 nm: (▼) transient absorption at zero time and (×) transient absorption spectrum of the final long-lived photoproduct. Inset: zoom in the 500−800 nm region of the spectrum.

another species (Figure 7, ×). This decay process is monoexponential and has a lifetime of 84 μs.

The transient absorption spectrum of the successor state demonstrates a rather long lifetime (350 μs) and is characterized by the maximum at 700 nm, a weaker maximum at 480 nm, and a shoulder at 650 nm (inset of Figure 7, ×). These features agree very well with the transient spectrum of the $ZnTMPyP^{4+}$ radical cation obtained by the pulse radiolysis technique albeit at pH 7 and under different conditions.[31] This suggests that in the presence of 15 equiv of laccase the long-lived radical cation of $ZnTMPyP^{4+}$ forms from the triplet state of the porphyrin. Hence the oxidation of the photoexcited porphyrin is undoubtedly due to the redox photoreaction with laccase. The yield of the charge-separated state formation between the triplet of $ZnTMPyP^{4+}$ ($^3ZnP^*$) and laccase was estimated from the transient spectra shown in Figure 7 using three complementary approaches. These were based on (*i*) the intensity of the transient absorption of porphyrin triplet at 470 nm and that of porphyrin cation at 700 nm; (*ii*) the intensity of ground-state bleach of Q(0,1) absorption band of the porphyrin at 570 nm for the initial triplet state with respect to that for the formed radical cation; and (*iii*) the intensity of the bleach of the porphyrin Soret band at 430 nm for both initially formed porphyrin triplet and the formed porphyrin cation which persisted at longer times. In the first case, the concentrations of both the initial porphyrin triplet molecules and formed porphyrin radical cations were calculated using available extinction coefficients: 1×10^4 M^{-1} cm^{-1} at 700 nm for the $ZnTMPyP^{4+}$ cation[31] and 7×10^4 M^{-1} cm^{-1} at 470 nm for the $ZnTMPyP^{4+}$ triplet state (assuming an extinction coefficient similar to that of zinc tetraphenylporphyrin (ZnTPP)).[29] In the two latter cases, the estimate was calculated directly from the ratio of the corresponding bleach signal for the porphyrin radical cation spectrum (× in Figure 7) and that for the initial porphyrin triplet spectrum (▼ in Figure 7). The average value of the yield of charge separation between the porphyrin triplet state and laccase leading to the radical cation was estimated as 0.35 ± 0.10.

DISCUSSION

White light irradiation induces laccase excitation with a concomitant slight reduction of the Cu(II) T1 ion observable within tens of minutes in the absence of dioxygen. According to the work of Henry and Peisach on MCOs including the laccase from *Rhus vernicifera* (a plant laccase), this reduction is mainly attributable to the excitation of the Cu(II) T3 pair of the TNC at λ = 330 nm.[32] With the addition of the $ZnTMPyP^{4+}$ photosensitizer and a sacrificial electron donor both the laccase Cu(II) T1 and T2 ions are reduced within minutes as evidenced by the loss of their ESR signals that are recovered upon reoxygenation of the samples. Since the reoxidation of the enzyme occurs only through an interaction of dioxygen with a fully reduced T3 pair,[33] it is possible to conclude that all the copper ions of the enzyme are fully reduced before air is let into the sample. In the presence of air an active laccase converts O_2 into water and indeed we observe an enzyme accomplishing full catalytic cycles in an irradiation-dependent way.

Excitation of $ZnTMPyP^{4+}$ generates a triplet excited state with a high quantum yield (ϕ_T = 0.9).[27,30] The lifetime of the triplet excited state of the porphyrin (τ_0 = 0.72 ms under our experimental conditions) is within the range reported by others.[34] In the absence of dioxygen, laccase quenches the $^3ZnP^*$ (τ = 84 μs) to generate a long-lived (~350 μs) radical cation of the porphyrin, $ZnP^{\bullet+}$, with a bimolecular quenching rate constant $k_Q \approx 1.4 \times 10^8$ M^{-1} s^{-1} obtained from the Stern−Volmer equation (eq 2).

$$\frac{\tau_0}{\tau} = k_Q \tau_0 [Q] + 1 \qquad (2)$$

where τ_0 and τ correspond to the lifetime of the triplet excited state in the absence and in the presence of the quencher (where Q is laccase), respectively, and k_Q is the quenching bimolecular rate constant. The quenching efficiency obtained from the lifetime ratio, τ_0/τ, is ~88%. However, the yield of charge separation estimated from the transient spectra is only 35% (*vide supra*). Therefore, only one out of approximately three quenching events in the sample leads to an efficient charge separation. Consequently, approximately two-thirds of the overall quenching might be due to a relaxation of the porphyrin triplet to the ground state. This relaxation may include different processes, most likely a nonradiative decay, where the excitation energy is transferred to the porphyrin and/or protein higher lying vibrational states and subsequently to the local environment.

Laccase and $ZnTMPyP^{4+}$ are charged species, and therefore electrostatic interactions might influence the photoinduced ET process. The stoichiometry of formation and binding constants as well as a photoinduced ET in electrostatically stabilized self-associated complexes involving porphyrins is well-established.[35] For example, in the case of plastocyanin, where an ion-pair complex is known to form between the cationic $ZnTMPyP^{4+}$ and the negatively charged protein, significant changes in the UV/vis spectrum of the porphyrin (10 nm shift of the Soret band) have been observed in the presence of rather low amounts of protein (i.e., 0.5 μM), allowing the determination of a binding constant of 3×10^6 M^{-1}.[35h] Laccases contain three plastocyanin-related domains (CUP domains, see Figure 1), and although a docking site identical to that found in plastocyanin is not present due to the domains 3D

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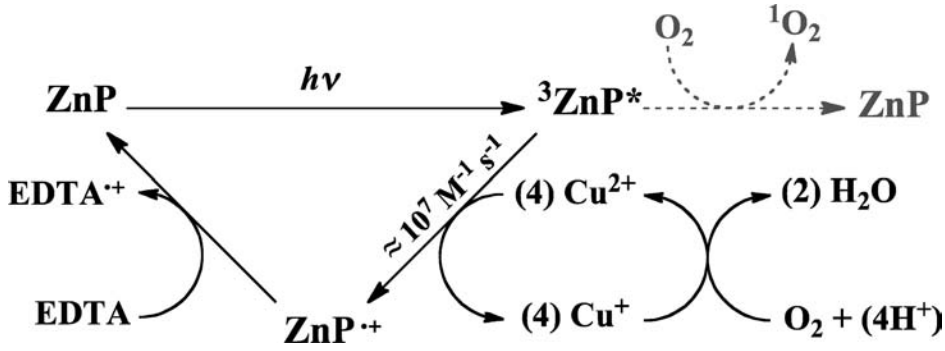
Scheme 1



organization, negatively charged amino-acids may potentially form patches at the surface of the enzyme. Kurzeev et al. recently observed an efficient ET (~10^8 M^{-1} s^{-1}) between a positively charged (i.e., +1) cyclometalated Ru(II) complex used as a redox mediator and a *Coriolus hirsuta* laccase. In that case they concluded both that ET is not dependent on ionic pairing and that the relative orientation of the partners does not define a binding site *per se*.[14b] While the charge distribution at the surface of the *C. hirsuta* and LAC3 laccases as well as the net charge (+1 vs +4) and the nature of the electron donors employed differ between the two systems, it seems that ion pairing might not be affecting ET efficiencies in the case of $ZnTMPyP^{4+}$/laccase either. Indeed, only a small shift in the Soret band (≤2 nm) observed upon laccase addition in steady-state photolysis experiments suggests a rather weak interaction between $ZnTMPyP^{4+}$ and laccase. Moreover, only slight changes in the Soret band of the $ZnTMPyP^{4+}$ were observed upon titrating the porphyrin with laccase (Figure SI3). These changes were observable only with fairly high concentrations of laccase, making the calculation of a binding constant meaningless. Therefore, the ET process in our system can be primarily described through collisions between the sensitizer and the enzyme.

Upon excitation of $ZnTMPyP^{4+}$ in the presence of the enzyme, the large driving force induced by the reducing power of the excited state of the $ZnTMPyP^{4+}$ is in favor of the oxidation of the sensitizer ($E°(ZnP^{\bullet+}/^3ZnP^*)$ = −0.45 V vs. SHE, $E°(Cu^{2+}/Cu^+)$ = 0.68 V for the T1 copper).[30,14a] Upon reduction of the blue copper site, the transient spectrum of the photoproducts must contain a contribution proportional to the amount of Cu(II) transiently reduced. However, laccase has rather low molar extinction in this region of approximately (5−6 × 10^3 M^{-1} cm^{-1}). This value is much lower than that of the porphyrin Q(0,1) band ($\varepsilon \approx 16 \times 10^3$ M^{-1} cm^{-1} maximum at 560 nm) and that of the porphyrin radical cation, which has absorption at ~700 nm ($\varepsilon \approx 10 \times 10^3$ M^{-1} cm^{-1}). Moreover, the weakest porphyrin Q(0,0) absorption band has maximum at 602 nm and $\varepsilon \approx 5 \times 10^3$ M^{-1} cm^{-1}, similar to that of laccase. Given the extinction coefficients and spectral positions, it appears hardly possible to resolve a weak bleach of the laccase on the background of much stronger porphyrin transient signals in this very congested spectral region. In addition to the blue copper site, an involvement of a surface-accessible amino acid as an alternate electron-acceptor site is possible. In several multicopper proteins it has been shown that the ET process may, in some circumstances, also involve surface-accessible protein cystine disulfide residues (RSSR).[36] The T1 Cu(II) site of these proteins is thus eventually reduced through both an intermolecular process and an intramolecular process.[36] Disulfide radical anions $RSSR^-$ have a distinct and relatively intense absorption band at ~410 nm ($\varepsilon \approx 10 \times 10^3$ M^{-1} cm^{-1}). However, this spectral region is overwhelmed by the more intense porphyrin Soret band. On the other hand, it is noteworthy that the $RSSR^-$ radical has only been reported to form in the presence of strongly reducing radicals such as the pulse radiolytically generated CO_2^- radical,[36] the reducing potential of which ($E°(CO_2/CO_2^-)$ ≈ −1.8 to −2.0 V vs SHE[36,37]) is at least 1.35 V more negative than that of the radical cation of the porphyrin ($E°(ZnP^{\bullet+}/^3ZnP^*)$ = −0.45 V vs SHE[30]).

The rate constant of the CT reaction, k_{ET} = 5.5 × 10^7 M^{-1} s^{-1}, calculated from the equation (eq 3) is in the range of rate constants previously reported for bimolecular reactions between MCOs and flavins,[13] transition metal complexes,[14] radicals (e.g., O_2^- or CO_2^-),[12] Ru(II) bipyridyl sensitizers,[15] or cytochrome *c*[38] (from 10^4 to 10^9 M^{-1} s^{-1}),

$$\frac{\phi_{ET}}{[Q]\tau} = k_{ET} \qquad (3)$$

where k_{ET} is the electron transfer rate constant and ϕ_{ET} the charge separation yield. In contrast, the rate constant reported for ET between the tyrosine (Y83) residue of plastocyanin and the S_1 state of $ZnTMPyP^{4+}$ within their self-associated complex is several orders of magnitude higher.[35h]

Addition of EDTA results in the regeneration of the initial state ZnP by reduction of the $ZnP^{\bullet+}$ produced during the CT reaction (Scheme 1). As EDTA is not known to be a good quencher of the $^3ZnP^*$,[27] $ZnP^{\bullet-}$ is probably not a major quenching product obtained in EDTA-containing solution, even at [EDTA] representing 1000 equiv of [ZnP]. However, as is suggested by the irreversible bleaching of the ZnP Soret band observed in the steady-state photolysis, production of a small amount of $ZnP^{\bullet-}$ due to $^3ZnP^*$ reduction by EDTA cannot be excluded. In the course of ET quenching of $^3ZnP^*$, laccase is reduced stepwise by four electrons (*vide supra*). Similar to other laccases, the reduction of the T1 copper ion by a substrate is transient and leads to a partial reduction of the TNC by an intramolecular ET. As measured, this intramolecular ET is slow due to the absence of dioxygen.[39] In the presence of dioxygen, $^3ZnP^*$ can be quenched not only by laccase but also by O_2 to form singlet oxygen $O_2(^1\Delta_g)$ in an energy transfer process and O_2^- in an oxidative process, although the formation of the latter is probably largely compensated by a fast back electron transfer. Laccase-dependent dioxygen consumption (i.e., the rate of O_2 consumption sensitive to the inhibitor azide) is, however,

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reaching a value that is at least 4 times larger than the enzyme-independent one, highlighting the influence of the enzyme in the whole process.

In the present photocatalytic system, the photodriven laccase operates the reduction of dioxygen into water with turnovers up to 2.0 min^{-1} (corresponding to 8.0 min^{-1} for the one-electron oxidation process). This is a substantial photocatalytic performance in the range of those recently reported for hybrid systems made for either the reduction of CO_2[16b] or that of HCN.[17b] Notably, the overall global efficiency of the $ZnTMPyP^{4+}$/laccase bimolecular system presented here is substantially higher than the Ru(II) bipyridyl-type complexes/laccase systems that we have described previously.[15] Thus, with a 1:1 sensitizer/enzyme ratio, the maximum activity of the $ZnTMPyP^{4+}$/laccase system is 1 order of magnitude higher than that of the Ru(II) bipyridyl-type complexes/laccase systems. Moreover, the activity of the $ZnTMPyP^{4+}$/laccase system with a photosensitizer/enzyme ratio of 0.1 is 2−5 times higher than that obtained with a Ru(II) bipyridyl type complexes/laccase ratio at 1:1. We are aware that (*i*) such a macroscopic effect probably results from a combination of factors and (*ii*) photophysical properties of the two sensitizers are quite different. Nevertheless, some analogies and/or differences can be safely highlighted. The reduction of the acceptor (the laccase) is thermodynamically favored for both systems and both systems function apparently through a collision mode. There is a marked difference in the calculated bimolecular rate constant for the ET from quantitatively generated excited state of the photosensitizers to the enzyme, namely $\sim 10^7$ M^{-1} s^{-1} for the $^3ZnP^*$ and $\sim 10^9$ M^{-1} s^{-1} for the $Ru^{II}*$. On the other hand, the lifetime of the triplet excited state of $ZnTMPyP^{4+}$ is ∼3 orders of magnitude longer than that of the $^3[Ru(bpy)_3^{2+}]$ under similar experimental conditions (720 μs vs 600 ns). Assuming the same reaction pathway for the bimolecular ET, the longer lifetime of the $^3ZnP^*$ photosensitizer will lead to more efficient quenching of its excited state by laccase if compared to the same process with $^3[Ru(bpy)_3]^{2+}$. Consistent with this assumption, as estimated from the transient spectra, the yield of redox products formed with the $ZnTMPyP^{4+}$/laccase system is $\phi_{ET} = 0.35$, whereas it is $\phi_{ET} = 0.08$ (derived from eq 3) with the $Ru(bpy)_3^{2+}$/laccase system.

CONCLUSION

Overall, assembling a sensitizer and a multicopper oxidase in a new type of photocatalytic system link the oxidation of an electron donor to the four-electron reduction of dioxygen. Avoiding the use of noble or rare metals using a Zn porphyrin as a photosensitizer, we have achieved a substantial photocatalytic dioxygen reduction. The efficiency of the system is primarily dependent on the photophysical properties of the sensitizer. Therefore, a quest for alternative sensitizers may allow one to improve the efficiency of the system. Optimization of the system may also lie in the covalent attachment of the photosensitizer to the enzyme in a photocatalytic hybrid. To this end, mono-derivatization of a unique residue near the T1 copper site in laccase mutants is in progress in our laboratory. Finally, the substantial dioxygen reduction achieved with the $ZnTMPyP^{4+}$/laccase system is concomitant to a substantial aerobic oxidation of an electron donor. Therefore, this work has made plausible the idea of aerobic photodriven transformations on substrates of interest using a hybrid photocatalyst incorporating an enzyme and a photosensitizer.

ASSOCIATED CONTENT

Supporting Information

Dioxygen consumption dependence on [laccase] or $[ZnTMPyP^{4+}]$ in the presence or absence of sodium azide; stopped-flow; electronic ground state absorption; global fitting of the flash photolysis data; titration of $ZnTMPyP^{4+}$ by laccase. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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ϕ_{ET} in the last sentence in the Discussion was corrected on February 19, 2013.