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Photoinduced Multielectron Transfer to a Multicopper Oxidase Resulting in Dioxygen Reduction into Water

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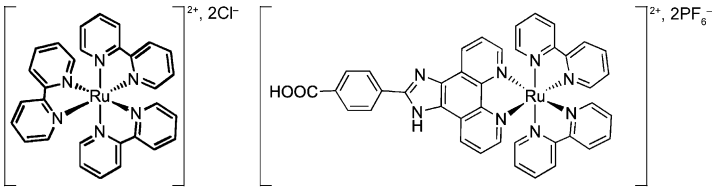
By mimicking the naturally evolved enzymatic and photochemical processes of photosynthesis, solar energy can be used to drive catalysis and ultimately convert light to stored chemical energy.[1] The target is to develop robust systems in which light absorption triggers electron-transfer events that subsequently lead to the activation of a catalytic center. Such a process would not only avoid chemical activation by harsh oxidants or reductants, one particular focus of green chemistry, but also diminish the dependence on nonrenewable energy sources.[2] In addition to these now well-established goals of sustainable energy and resource use, the development of photodriven catalysts potentially offers new avenues for the study of catalytic mechanisms. In this field Gray, Winkler, and co-workers pioneered the coupling of photoactive units to enzymes to access the buried active site of different metalloenzymes.[3]

The well-documented Ru^{II}-polypyridine-type complexes have been used to initiate electron-transfer reactions in the presence of either sacrificial electron acceptors or electron donors for the oxidation or reduction of active sites.[4] One challenging issue when using this strategy to activate metalloenzymes, is the efficient accumulation of multicharges or holes at the catalytic unit. In the majority of cases light-induced transfer processes have been limited to one electron transfer. Only a few examples of multicharge or hole accumulations have been reported recently.[5] Some rare examples using metalloproteins that could lead to photodriven catalytic activities were also reported.[6] Herein, we report the light-driven four-electron reduction of a laccase (our previously studied LAC3 from *Trametes* sp. C30[7]), which ultimately converts dioxygen into water by using ruthenium(II) polypyridine-type chromophores (complexes **1** and **2**, Scheme 1) and ethylenediaminetetraacetic acid (EDTA) as the sacrificial electron donor.



1 2

Scheme 1. Ruthenium complexes used in this study.

Laccase is a multicopper oxidase that naturally accumulates four electrons (from successive one-electron oxidations of phenolic substrates) on four copper ions and in turn reduces dioxygen to water.[8] Laccase contains a surface-located type 1 (T1) copper center (substrate oxidation center) connected to a trinuclear center (TNC, dioxygen reduction center) structured between a type 3 (T3) pair of antiferromagnetically coupled copper ions, and a type 2 (T2) copper ion. Laccase-catalyzed oxidations of transition-metal complexes that illustrate the ability of the enzyme to interact with such partners have been reported.[9]

Complex **2** is a heteroleptic ruthenium(II) complex with one of the ligands holding a fused imidazole motif and a substituted phenyl ring reminiscent of the substrates of laccases.[10] While challenging the oxidation of syringaldazine (4-hydroxy-3,5-dimethoxybenzaldehyde azine, a commonly used substrate of laccases) by LAC3 with several concentrations of **2**, we found that **2** inhibits LAC3 with an IC50 of about 200 μM. $NaPF_6$ has no noticeable effect on the enzyme activity up to 200 μM. This indicates that complex **2** can probably interfere with the binding site of the substrate. In contrast, in the presence of 300 μM of **1**, LAC3 displayed approximately 90% of the residual activity.

Photoreduction of LAC3 was first followed by UV/Vis spectroscopy. The spectrum of LAC3 displays the character-

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istic features of T1 copper sites with an intense absorption band centered at 610 nm ($\varepsilon = 5600\ mol^{-1}\,L\,cm^{-1}$) responsible for the blue color of the oxidized enzyme.[8] Under irradiation and in the presence of **2** (or **1**) and EDTA, the 610 nm band was bleached within a few minutes, which indicated a reduction of the T1 Cu^{II} ion to Cu^{I} (Figure 1). This suggests that at least one electron was transferred to the enzyme under illumination. Irradiation experiments performed in the absence of EDTA and/or the Ru^{II} complex did not lead to significant reduction of the T1 Cu^{II} in the same timescale (Figure S1 in the Supporting Information). On the other hand, although known as a metal chelator, we verified that EDTA does not affect LAC3 activity (see Figure S2 in the Supporting Information).

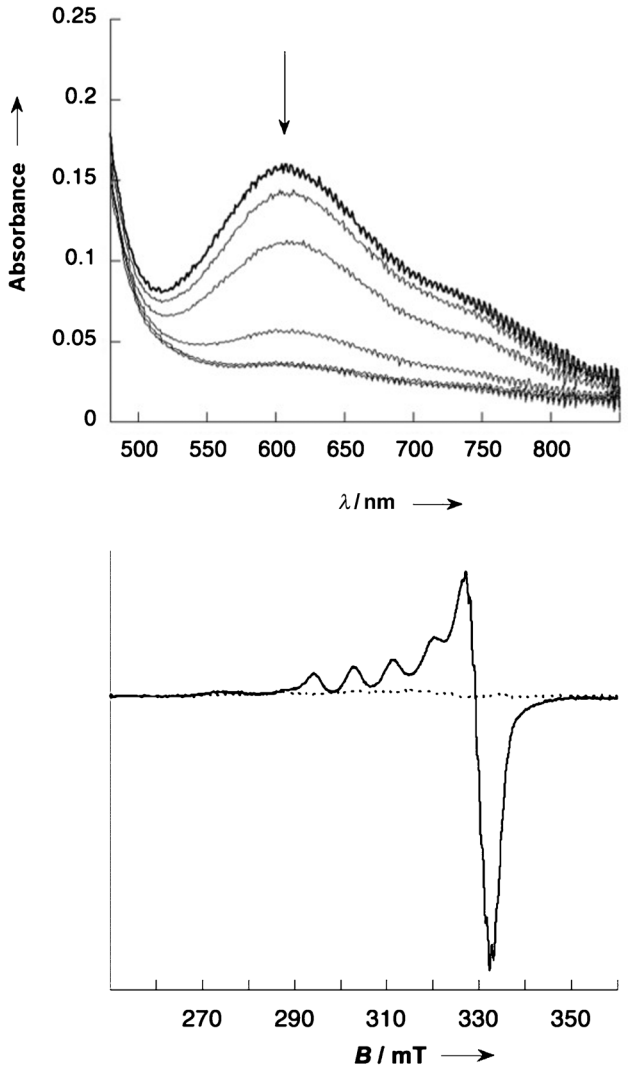

Figure 1. Evolution of the spectroscopic features of LAC3 in the presence of complex **2** (1 equiv) and EDTA (50 equiv), under white light irradiation, at pH 5.5 under an inert atmosphere. Top: UV/Vis spectra with LAC3 (30 μM). Bottom: X-band ESR spectra recorded using 490 μM of laccase; before irradiation (——) and after irradiation (-----). Temperature 115 K, microwave power 20 mW, modulation 3G, gain 10^5.

To get quantitative data on electron-transfer rates from the photosensitizer to the T1 copper site, the luminescence lifetime of the light-excited complexes **1** and **2** (**1*** and **2***) were measured following a previously described procedure.[11] After irradiation at 355 nm, the luminescence at 605 nm follows a single exponential decay. In the absence of dioxygen, the ruthenium(II) complexes display excited-state lifetimes of 625 and 900 ns for **1*** and **2***, respectively, which are in agreement with the previously reported values.[4,12] In the presence of dioxygen, these lifetimes drop to 401 and 505 ns, respectively. The luminescence was quenched by adding increasing amounts of laccase, and bimolecular quenching rate constants were determined from Stern–Volmer plots. The excited state of Ru^{II} complexes may be quenched by an energy transfer and/or an electron transfer from the Ru^{II}* to the T1 Cu^{II} of LAC3 (oxidative quenching). Quenching of **1*** or **2*** by nonradiative energy transfer was evaluated to contribute by less than 5% (Figure S3 in the Supporting Information). Therefore, the quenching rate constants are taken as the electron-transfer rate constant from the Ru^{II}* complexes to the Cu^{II} T1. The Stern–Volmer plots for complex **1** are linear up to 150 μM (Figure S4 in the Supporting Information). A slight deviation from linearity is detectable for complex **2** at concentrations of 130–150 μM of LAC3 and could indicate an association of the complex to the protein as supported by the kinetic data (Figure 2). Further studies are under way to characterize a possible association of complex **2** to the enzyme. When experiments are handled at pH 5.5 in aerobic conditions, k_{ET} are 0.81×10^9 and $0.65\times10^9\ mol^{-1}\,L\,s^{-1}$ for complexes **1** and **2**, respectively. However, in the absence of dioxygen, for a not yet understood reason, the rate constants are approximately three times larger (Table 1). The values reported here are consistent with the rate constants reported for the quenching of $Ru(bpy)_3^{2+}$* by the oxidized T1 copper ions of several blue

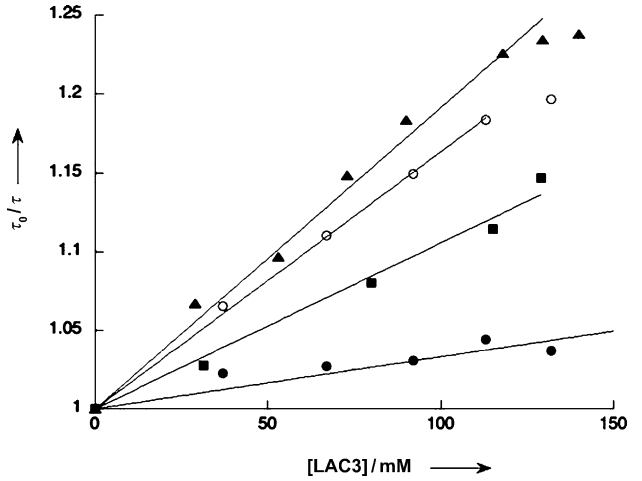

Figure 2. Stern–Volmer plots for the quenching of the luminescence of **2*** by LAC3 at 20 °C: ▲ = pH 6 under nitrogen, ■ = pH 3 under nitrogen, ○ = pH 5.5 under nitrogen and ● = pH 5.5 in air. τ_0: excited-state lifetime in the absence of laccase, and τ : excited-state lifetime in the presence of laccase. ($\lambda_{exc} = 355$ nm and $\lambda_{em} = 605$ nm).

Table 1. Electron-transfer rate constants from Ru^{II}* complexes to the T1 Cu^{II} under nitrogen unless specified, and dioxygen consumption rates.

	pH	$k_{ET}\times10^{-9}$ [$mol^{-1}Ls^{-1}$]	$V_{O2}\times10^{6}$ [$molL^{-1}min^{-1}$] +LAC[b]	−LAC[c]
1	3.0	1.88 ± 0.06	8.1	3.2
	5.5	$2.48\pm0.05/0.81\pm0.01$[a]	nd	nd
	6.0	2.55 ± 0.03	6.6	5.4
	9.0	nd	6.4	13.3
2	3.0	1.14 ± 0.04	7.8	2.7
	5.5	$1.81\pm0.02/0.65\pm0.01$[a]	nd	nd
	6.0	2.06 ± 0.05	8.1	5.8
	9.0	nd	8.8	15.0

[a] Under O_2. [b] EDTA + Ru^{II} complex + LAC3. [c] No LAC3.

copper proteins,[13] as well as with that reported for the photoreduction of ascorbate oxidase by photolytically generated 5-deazariboflavin semiquinone.[14] In any case, electron-transfer rates appear to be fast approaching the limit of diffusion-controlled processes. The high reducing power of the excited state of the Ru^{II} complexes, inducing a large driving force ($E°(Ru^{3+}/Ru^{2+}*)=-0.84$ and -0.46 V versus NHE for **1** and **2**, respectively, $E°(Cu^{2+}/Cu^{+})=0.68$ V for the T1 copper),[4,9a,12] may partially account for this phenomenon.

The photoreduction process was further monitored by ESR spectroscopy. The sample (LAC3 + ruthenium complex + EDTA) was placed in an ESR quartz tube. Prior to irradiation, the 115 K spectrum of LAC3 (Figure 1) was composed of signals arising from the T1 and T2 ions with parameters for the T1 ($g_{\parallel}=2.19$; $A_{\parallel}=87$ G; $g_{\perp}=2.04$) and for the T2 ($g_{\parallel}=2.25$; $A_{\parallel}=162$ G), similar to those for LAC3 and other laccases reported previously.[7,8] After approximately 25 min of irradiation the sample was ESR-silent, which indicated that both the T1 and T2 coppers were reduced. This formally indicates that at least two electrons were transferred to the enzyme. Upon reoxygenation, both the T1 and T2 coppers were reoxidized as assessed by the reappearance of the UV/Vis and ESR features. Direct oxidation of the T1 copper by dioxygen does not occur in multicopper oxidases, and O_2 interacts instead on the reduced T3 pair.[15,16] The reoxidation of both T1 and T2 copper centers, thus, supports the fact that the T3 copper atoms were also reduced after irradiation. Cycles of anoxic photoreduction followed by reoxidation of the copper centers did not alter the enzyme activity.

A fully reduced 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 3.0, 6.0, or 9.0. In the dark, in the absence of photosensitizer, or when complexes **1** or **2** were used alone, no significant dioxygen consumption was measured. Upon irradiation of solutions containing 30 μM of **1** or **2** and 50 equivalents of EDTA with or without LAC3, the dioxygen concentration decreased. Light-induced dioxygen consumption rates, in the presence or the absence of LAC3, range from about 3 to 15×10^{-6} $molL^{-1}min^{-1}$ and are very similar for the two complexes (Table 1).

The Ru^{II} excited state can be quenched by O_2 to form 1) singlet oxygen $O_2(^1\Delta_g)$ in an energy-transfer process and 2) O_2^- in an oxidative process, although this is probably compensated by fast back electron transfer and does not account for a significant O_2 consumption.[17] When **1** or **2** were irradiated in the presence of EDTA, dioxygen was consumed in a pH-dependent process probably as the result of a complex mechanism. The reaction of EDTA with Ru^{III} to generate radical species is faster at basic pH values.[18] Therefore, highly reducing radicals derived from EDTA could be directly involved in the observed consumption of O_2. Although not favorable in the case of **1**,[18] a reductive quenching of Ru^{II}* by EDTA and an involvement of a Ru^{I} species in the dioxygen consumption cannot be excluded in the case of **2**. In the presence of LAC3, in addition to the above-mentioned possibilities, Ru^{II} excited states can be quenched by the enzyme as shown by luminescence lifetime experiments. At pH 3.0, O_2 consumption rates are 2.5 to 3 times higher in the presence than in the absence of laccase and appear to be dominated by an enzyme-dependent mechanism. Indeed, this extra consumption was completely removed by addition of NaN_3 (10 mM; Figure S3 A in the Supporting Information), a strong inhibitor interacting with the TNC of laccase.[19] At the same time, the presence of azide had no measurable effect on the dioxygen consumption rates in the absence of enzyme (Ru^{II}/EDTA systems, Figure S3 B in the Supporting Information). At pH 9.0 O_2 consumption rates, measured with **1** or **2** in the presence of LAC3, did not increase with pH, in contrast to the stimulating effect of pH observed in the absence of enzyme. Laccase is known to be rapidly inhibited by hydroxyl ions,[20] and, indeed, the enzyme is not able to reduce dioxygen at this pH. A strong inhibition of dioxygen consumption by azide and a dioxygen consumption faster at acidic than at alkaline pH, support the fact that, under irradiation, the photoreduced enzyme can accomplish full catalytic cycles during which four electrons per cycle are stored on the copper ions.

In conclusion, we have described for the first time the photoinjection of four electrons into a multicopper oxidase. In the presence of an exogenous electron donor and a photosensitizer, the enzyme can be fully reduced and is in turn able to reduce dioxygen. Although the turnover of this bimolecular system remains modest ($0.3\ min^{-1}$), it constitutes a first step towards promising hybrid materials. Indeed, one way to improve the system is to link the photosensitizer to the enzyme covalently. To this end, construction of site-directed mutants with a unique anchor point near the T1 copper site is in progress in our laboratory. Moreover, given the availability of various photosensitizers and sacrificial electron donors/acceptors, as well as the plasticity of laccases, it is likely that a great diversity of hybrid materials with applications in the fields of photocatalysis, photonics, and energy can be created.

Experimental Section

Complex **1** was purchased from Aldrich, and complex **2** was prepared by modification of previously described procedures (see the Supporting Information for details).[9] The photoinduced electron-transfer steps were performed in sealed vessels containing argon- or nitrogen-flushed Britton–Robinson (B&R) buffer at the appropriate pH using LAC3 with 1 equivalent of ruthenium complex and 50 equivalents of EDTA. Irradiations were performed with a 150W Xenon bulb (Dolan-Jenner MI-150 fiber-optic illuminator). These reaction conditions were not deleterious to the enzyme, as assessed by gel electrophoresis and enzyme activity measurements.

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[1] K. Sanderson, *Nature* **2008**, *452*, 400–402.
[2] T. Collins, *Science* **2001**, *291*, 48–49.
[3] a) J. J. Wilker, I. J. Dmochowski, J. H. Dawson, J. R. Winkler, H. B. Gray, *Angew. Chem.* **1999**, *111*, 93–96; *Angew. Chem. Int. Ed.* **1999**, *38*, 89–92; b) H. B. Gray, J. R. Winkler, *Biochim. Biophys. Acta Bioenergetics* **2010**, *1797*, 1563–1572.
[4] a) K. Kalyanasundaram, *Coord. Chem. Rev.* **1982**, *46*, 159–244; b) J. G. Vos, J. M. Kelly, *Dalton Trans.* **2006**, 4869–4883.
[5] a) S. Fukuzumi, *Phys. Chem. Chem. Phys.* **2008**, *10*, 2283–2297; b) R. Konduri, NR. De Tacconi, K. Rajeshwar, F. M. MacDonnell, *J. Am. Chem. Soc.* **2004**, *126*, 11621–11629; c) S. Karlsson, J. Boixel, Y. Pellegrin, E. Blart, H. C. Becker, F. Odobel, L. Hammarström, *J. Am. Chem. Soc.* **2010**, *132*, 17977–17979.
[6] a) L. E. Roth, J. C. Nguyen, F. A. Tezcan, *J. Am. Chem. Soc.* **2010**, *132*, 13672–13674; b) E. Reisner, D. J. Powell, C. Cavazza, J. C. Fontecilla-Camps, F. A. Armstrong, *J. Am. Chem. Soc.* **2009**, *131*, 18457–18466; c) T. W. Woolerton, S. Sheard, E. Reisner, E. Pierce, S. W. Ragsdale, F. A. Armstrong, *J. Am. Chem. Soc.* **2010**, *132*, 2132–2133.
[7] A. Klonowska, C. Gaudin, M. Asso, A. Fournel, M. Réglier, T. Tron, *Enz. Microb. Technol.* **2005**, *36*, 34–41.
[8] E. I. Solomon, R. K. Szilagyi, S. DeBeer George, L. Basumallick, *Chem. Rev.* **2004**, *104*, 419–458.
[9] a) V. Balland, C. Hureau, A. M. Cusano, Y. Liu, T. Tron, B. Limoges, *Chem. Eur. J.* **2008**, *14*, 7186–7192; b) S. A. Kurzeev, A. S. Vilesov, T. V. Fedorova, E. V. Stepanova, O. V. Koroleva, C. Bukh, M. J. Bjerrum, I. V. Kurnikov, A. D. Ryabov, *Biochemistry* **2009**, *48*, 4519–4527.
[10] a) F. Lachaud, A. Quaranta, Y. Pellegrin, P. Dorlet, M.-F. Charlot, S. Un, W. Leibl, A. Aukauloo, *Angew. Chem.* **2005**, *117*, 1560–1564; *Angew. Chem. Int. Ed.* **2005**, *44*, 1536–1540; b) A. Quaranta, F. Lachaud, C. Herrero, R. Guillot, M.-F. Charlot, W. Leibl, A. Aukauloo, *Chem. Eur. J.* **2007**, *13*, 8201–8211; c) C. Herrero, J. L. Hughes, A. Quaranta, N. Cox, A. W. Rutherford, W. Leibl, A. Aukauloo, *Chem. Commun.* **2010**, *46*, 7605–7607.
[11] J. Zakrzewski, J. A. Delaire, C. Daniel, I. Cote-Bruand, *New J. Chem.* **2004**, *28*, 1514–1519.
[12] Y. Pellegrin, R. J. Forster, T. E. Keyes, *Inorg. Chim. Acta* **2009**, *362*, 1715–1722.
[13] B. S. Brunschwig, P. J. Delaive, A. M. English, M. Goldberg, S. L. Mayo, N. Sutin, H. B. Gray, *Inorg. Chem.* **1985**, *24*, 3743–3749.
[14] L. Santagostini, M. Gulloti, J. T. Hazzard, S. Maritano, G. Tollin, A. Marchesini, *J. Inorg. Biochem.* **2005**, *99*, 600–605.
[15] N. J. Blackburn, M. Ralle, R. Hassett, D. J. Kosman, *Biochemistry* **2000**, *39*, 2316–2324.
[16] N. Hakulinen, L.-L. Kiiskinen, K. Kruus, M. Saloheimo, A. Paananen, A. Koivula, J. Rouvinen, *Nature Struct. Biol.* **2002**, *9*, 601–605.
[17] Q. G. Mulazzani, H. Sun, M. Z. Hoffman, W. E. Ford, M. A. J. Rodgers, *J. Phys. Chem.* **1994**, *98*, 1145–1150.
[18] M. Z. Hoffman, *Inorg. Chem.* **1989**, *28*, 978–980.
[19] I. Gromov, A. Marchesini, O. Farver, I. Pecht, D. Goldfarb, *Eur. J. Biochem.* **1999**, *266*, 820–830.
[20] F. Xu, W. S. Shin, S. H. Brown, J. A. Wahleithner, U. M. Sundaram, E. I. Solomon, *Biochim. Biophys. Acta Protein Struct. Mol. Enzymol.* **1996**, *1292*, 303–311.

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