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Mechanism of Laccase–TEMPO-Catalyzed Oxidation of Benzyl Alcohol

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The oxidation of benzyl alcohol by air, catalyzed by the organocatalyst TEMPO and the enzyme laccase has been investigated. To establish the kinetically significant pathways and corresponding kinetic parameters, a series of experiments is conducted with synthesized stable oxidized and reduced forms of the organocatalyst, the oxoammonium cation, and hydroxylamine. The time course of TEMPO and its oxidized and reduced derivatives is monitored off line by a combination of GC analysis, UV/Vis spectroscopy, EPR spectroscopy, and FTIR spectroscopy. TEMPO is found to be regenerated through non-catalyzed comproportionation of the oxoammonium cation with hydroxylamine. A kinetic model is presented based on the experimentally determined kinetically significant pathways. The time dependences of the concentrations of the three redox states of TEMPO and benzyl alcohol are adequately described by the model. The results provide new leads for the development of a practical process for a combined laccase–TEMPO-catalyzed selective oxidation of alcohols.

Introduction

The oxidation of primary alcohols to the corresponding aldehydes and carboxylic acids plays a pivotal role in organic chemistry. Selective oxidation of primary alcohols is traditionally carried out using stoichiometric amounts of inorganic oxidants such as chromium(VI) salts.[1] Unfortunately, such procedures may lead to pollution of the environment and to health and safety risks, and hence needs a more efficient and cleaner alternative. Moreover, it is economically more attractive to use oxygen rather than an inorganic salt as the primary oxidant, and preferably oxygen from air under ambient conditions. Therefore, much research has been carried out towards the development of catalytic systems capable of selective primary alcohol oxidation based on oxidation by air.

An important step towards selective oxidation of primary alcohols has been the introduction of systems catalyzed by the organocatalyst 2,2,6,6-tetramethylpiperidinyl-1-oxyl (TEMPO), which is commercially available and stable in air. The oxidation of TEMPO yields the corresponding oxoammonium cation. This cation is capable of direct and selective oxidation of primary alcohols to yield the corresponding aldehydes, with concomitant reduction of the oxoammonium species to the hydroxylamine.[2] The catalytic cycle is closed by oxidation of the hydroxylamine to the oxoammonium species. Oxygen can be used as a primary oxidant in such a system, but homogeneous catalysts containing metals such as ruthenium or copper must be added to carry out the oxidation at a significant and suitable rate.[1,3] Recent work has shown that it is possible to replace these homogeneous catalysts with the enzyme laccase, a multi-copper oxidase, which is present in plants, fungi and bacteria and which is easily produced in large quantities.[4]

Laccases have been characterized structurally and functionally in detail, including the laccase from the fungus *Trametes versicolor* used in this work.[5–8] Laccases incorporate four copper atoms arranged at three copper centers: T1, T2, and the dicopper center T3. The T1, or blue, copper site serves as the initial electron acceptor (from TEMPO, for example). T1 transfers electrons to T2 and T3 possibly via a conserved His-Cys-His tripeptide electron-transfer pathway. The three copper atoms at T2 and T3 are only 3.8–3.9 Å apart and form a functional trinuclear copper site where molecular oxygen is reduced to water with transient formation of a peroxo/peroxide intermediate. Owing to the relatively low reduction potential (717 mV[5]) of the T1 center, laccase is not able to oxidize non-phenolic alcohols[9] but does oxidize TEMPO directly to oxoammonium.[5,10] Pilot experiments have shown that primary alcohols can indeed be oxidized using air and a bicatalytic system of laccase and TEMPO.[3,11] This latter study indicated that the rate of TEMPO regeneration by laccase is optimal at pH 4–5 and 30 °C in aqueous solution.

The individual reaction steps that may occur in the laccase–TEMPO system converting primary alcohols have been investigated intensively. These steps include alcohol oxidation by oxoammonium,[2,12–14] TEMPO oxidation by purified laccase,[5,15] and the comproportionation reaction between oxoammonium

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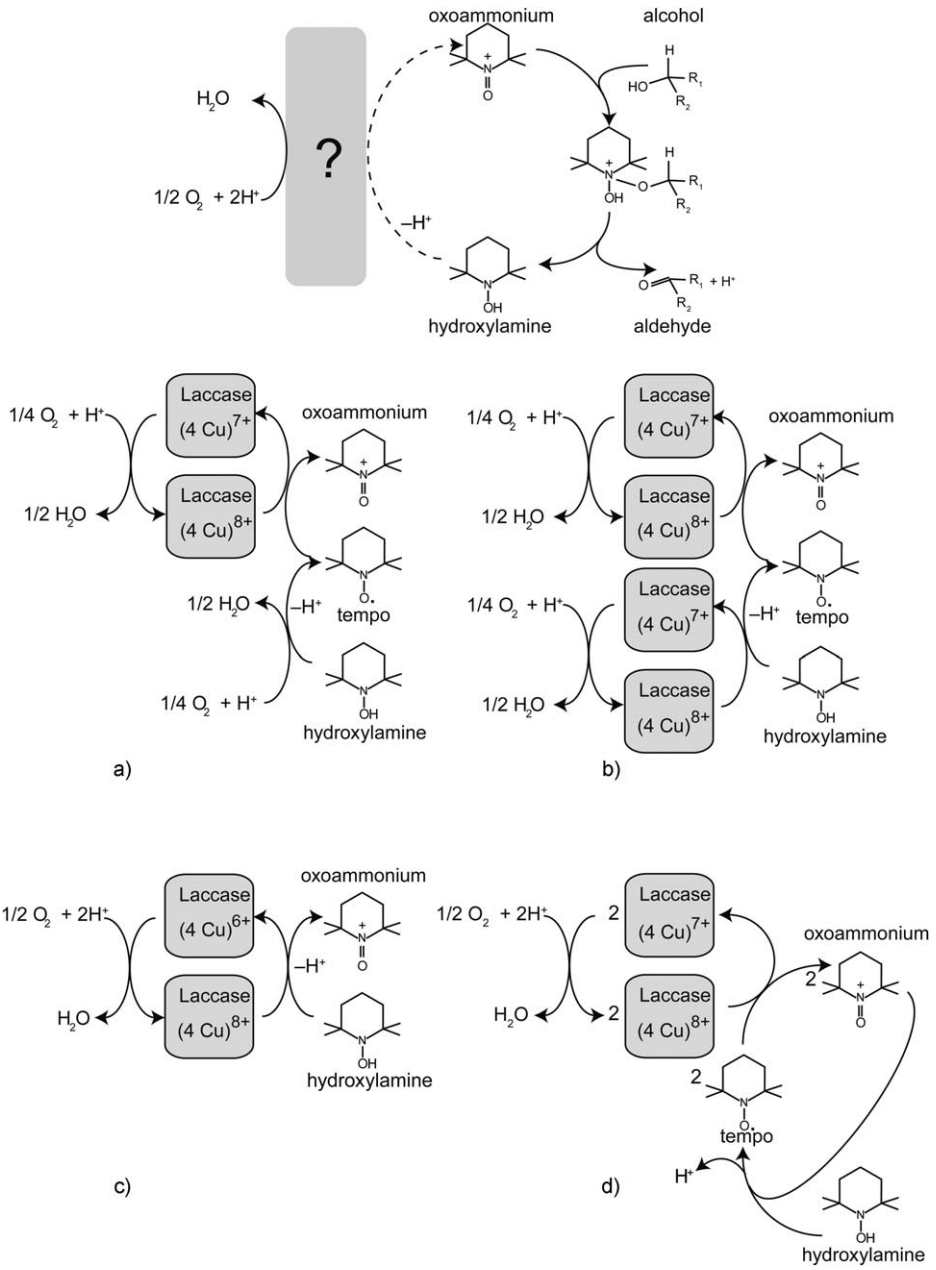
and hydroxylamine.[16] However, the key pathway and mechanism by which hydroxylamine is converted to oxoammonium are not known. To optimize the laccase–TEMPO system for the oxidation of primary alcohols, it is important to understand the reaction pathway and mechanism and to quantify the associated kinetic parameters. In this work, both the individual reaction steps and the overall reaction were studied. The time courses of the concentrations of TEMPO, its oxidized and reduced derivatives, and benzyl alcohol were determined by a combination of quantitative GC analysis, UV/Vis spectroscopy, EPR spectroscopy, and attenuated total reflectance (ATR) FTIR spectroscopy.[17] A quantitative model is presented that adequately describes alcohol conversion by the laccase–TEMPO system.



Scheme 1. Top) Overall reaction for alcohol oxidation by oxygen catalyzed by laccase and TEMPO, highlighting the unknown conversion from hydroxylamine to oxoammonium; a–d) The four kinetic routes considered for hydroxylamine oxidation: a) Direct noncatalytic oxidation of hydroxylamine to TEMPO by O_2, followed by single-electron oxidation of the formed TEMPO by O_2, catalyzed by laccase; b) single-electron oxidation of hydroxylamine to TEMPO by O_2 followed by a second single-electron oxidation to oxoammonium, both steps catalyzed by laccase; c) double-electron oxidation of hydroxylamine into oxoammonium by O_2, catalyzed by laccase; in this kinetic pathway, TEMPO is not a kinetically significant intermediate; d) nonenzymatic comproportionation of hydroxylamine with oxoammonium, followed by single-electron oxidation of the resultant TEMPO by O_2, catalyzed by laccase. $(4Cu)^{n+}$ ($n = 6$–8) indicates the total formal charge of the four copper ions, each being Cu^{2+} in the oxidized laccase, and with either one or two Cu^{2+} ions reduced to Cu^{1+} after reduction by TEMPO or hydroxylamine, respectively. Note, however, that the copper charges here are to conform to the chemical reaction stoichiometry making the reactions electroneutral. During turnover, all four copper centers are reduced, but not necessarily at the same time, to catalyze the reduction of oxygen to water.

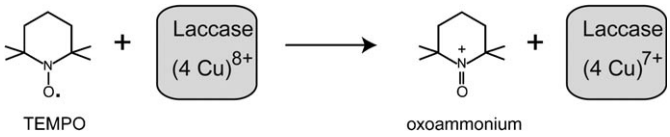
Results and Discussion

A main objective of this work is to determine the pathway for hydroxylamine oxidation to oxoammonium, in the presence of laccase and oxygen. Knowledge of this partial pathway can subsequently be used to understand and model the conversion of primary alcohols by the complete laccase–TEMPO system.

As shown in Scheme 1, four options for the oxidation of hydroxylamine have been considered. In pathways (a), (b), and (d), TEMPO is formed, which is oxidized with oxygen, catalyzed by laccase, to form an oxoammonium species.[5] Pathways (b) and (c) were experimentally assessed (data not shown) by adding hydroxylamine to the purified enzyme and monitoring reduction of the blue T1 copper site and also by oxygen consumption assays, both types of experiments suggesting a direct reaction between hydroxylamine and the enzyme. However, since the synthesized hydroxylamine was found to contain approximately 3 mol% TEMPO (for example, see Figure 3), these results were judged not conclusive. To determine more rigorously which of the four pathways is dominant, the various partial reactions discussed below were investigated.

TEMPO oxidation by laccase

The first reaction considered was the single-electron oxidation of TEMPO by laccase (Reaction 1; Scheme 2). The reaction rate is given by Equation (1).



Scheme 2. Reaction 1: Tempo oxidation by laccase; see legend to Scheme 1 for a note on the copper charges.

$$\frac{dC_{TEMPO}}{dt} = -r_1 = -k_1 \times C_{laccase} \times \frac{C_{TEMPO}}{K_{M,TEMPO} + C_{TEMPO}} \quad (1)$$

In Equation (1), C_{TEMPO} and $C_{laccase}$ are the molar concentrations of TEMPO and laccase, respectively, $K_{M,TEMPO}$ is the Michaelis–Menten constant for TEMPO, r_1 is the TEMPO reaction rate in м s^{-1} and k_1 is the enzymatic turnover number for the reaction in s^{-1}.The Michaelis–Menten constant was taken at 1.8×10^{-3} м, established in a previous study using the same fungal enzyme.[5]

The experimentally determined concentrations and the modeled concentrations of the different oxidation states of TEMPO for Reaction 1 (Scheme 2) are displayed in Figure 1.

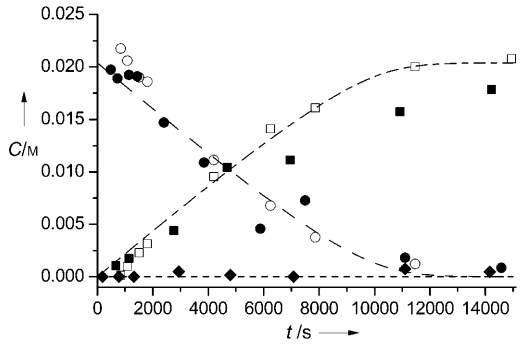
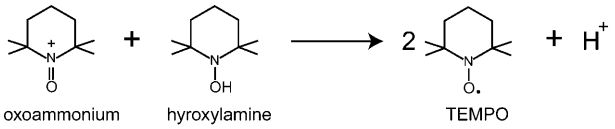


Figure 1. Time course of oxidation of TEMPO and formation of the products oxoammonium and hydroxylamine. The lines through the data points represent fits based on Reaction 1 (Scheme 2). See text and Table 2 for the fit parameters. Experimental conditions: Initial [TEMPO] = 20 мм, [laccase] = 0.67 μм, T = 30 °C, pH 4.3, under constant aeration. ● TEMPO (EPR); ○ TEMPO (UV/Vis); ◆ hydroxylamine (GC); ■ oxoammonium (IR); □ oxoammonium (UV/Vis); – – – TEMPO (model); ----- hydroxylamine (model); – – – oxoammonium (model).

The experimental stoichiometry of laccase-catalyzed TEMPO oxidation matches well with the theoretical stoichiometry of the reaction. The reaction is satisfactorily fitted with the Michaelis–Menten kinetics [Equation (1)]. The chosen analysis methods, GC and UV/Vis, EPR, and FTIR spectroscopy, provided adequate quantitative data. However, the oxoammonium concentration was slightly underestimated by FTIR spectroscopy, which can be attributed to sloping baselines. The turnover number k_1 for laccase was 3.6 s^{-1}, which is close to the value of 3.4 s^{-1} found under reactive conditions in previous work.[20]

Comproportionation reaction in the absence of laccase

The second reaction considered is the comproportionation reaction between oxoammonium and hydroxylamine in which two molecules of TEMPO and a proton are formed (Reaction 2; Scheme 3). The reaction rate is given by Equation (2).



Scheme 3. Reaction 2: Comproportionation reaction.

$$\frac{dC_{hydroxylamine}}{dt} = -r_2 = -k_2 \times C_{TEMPO} \times C_{oxoammonium} \quad (2)$$

In Equation (2), $C_{hydroxylamine}$, C_{TEMPO}, and $C_{oxoammonium}$ are the molar concentrations of hydroxylamine, TEMPO, and oxoammonium, respectively, r_2 is the rate of hydroxylamine conversion (м s^{-1}), and k_2 is the second-order rate constant ($м^{-1}s^{-1}$).

The experimentally determined concentrations and the modeled concentrations of the different oxidation states of TEMPO are shown in Figure 2. The experimental stoichiometry of Reaction 2 (Scheme 3) matched well with the theoretical stoichiometry. The reaction between oxoammonium and hydroxylamine was fitted well with a second order rate constant of 0.23 $м^{-1}s^{-1}$, which is in excellent agreement with the value

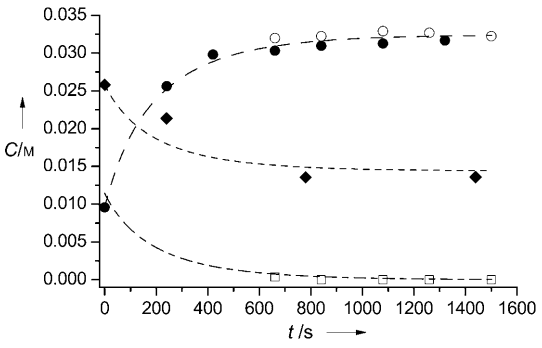


Figure 2. Time course of the comproportionation reaction and quantitative analysis of the concentrations of TEMPO, oxoammonium and hydroxylamine during the reaction. The lines through the data points represent fits based on Reaction 2 (Scheme 3). See text and Table 2 for the fit parameters. Experimental conditions: Initial [TEMPO] = 9.6 мм, [hydroxylamine] = 27.0 мм, [oxoammonium] = 11.0 мм, T = 30 °C, pH 4.3, under constant aeration. ● TEMPO (EPR); ○ TEMPO (UV/Vis); ◆ hydroxylamine (GC); □ oxoammonium (UV/Vis); – – – TEMPO (model); ----- hydroxylamine (model); – – – oxoammonium (model).

found by Israeli et al.[16] ($0.23\ M^{-1}s^{-1}$, found at pH 4.6). The good match between the theoretical and experimental reaction stoichiometries and between the rate constant determined here and by others and by other methods,[16] indicates that the chosen analysis methods can be applied quantitatively and, furthermore, that TEMPO and its derivatives are stable under the reaction conditions.

Hydroxylamine oxidation in the presence of laccase

The kinetic parameters found for Reactions 1 (Scheme 2) and 2 (Scheme 3) were used to determine and model the kinetic mechanism of hydroxylamine oxidation in the presence of laccase. The laccase concentration was varied by an order of magnitude (0.19 μM and 2.2 μM laccase, Figure 3) to investigate its effect on the reaction rate and to distinguish between alternative models.

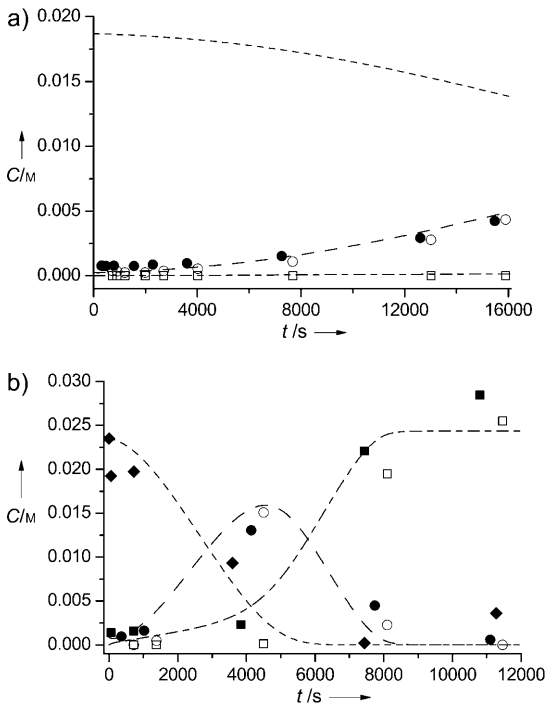
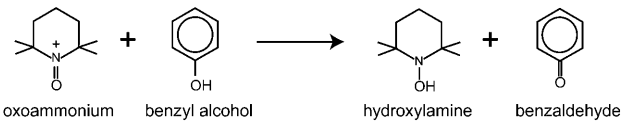


Figure 3. Time course of hydroxylamine oxidation in the presence of laccase at 0.19 μM (a) and 2.2 μM (b). The lines through the data points represent fits based on pathway (d) in Scheme 1. See text and Table 2 for the fit parameters. Experimental conditions: a) Initial [hydroxylamine] = 18.0 mM, [TEMPO] = 0.3 mM; b) initial [hydroxylamine] = 23.5 mM, [TEMPO] = 0.9 mM; other conditions as in Scheme 1. Note, for example, at $t = 0$, that the hydroxylamine stock may contain approximately 3 mol% TEMPO. ● TEMPO (EPR); ○ TEMPO (UV/Vis); ◆ hydroxylamine (GC); ■ oxoammonium (IR); □ oxoammonium (UV/Vis); ––– TEMPO (model); ----- hydroxylamine (model); – – – oxoammonium (model).

Oxidation in the presence of 0.19 μM laccase (Figure 3a) shows an increase in the rate of TEMPO formation with increasing TEMPO concentration, whereas, with a laccase concentration of 2.2 μM (Figure 3b), the amount of TEMPO formed goes through a maximum; furthermore, oxoammonium is determined as the final oxidation product. The total amount of organocatalyst remains constant during the reaction, indicating that the organocatalyst is not degraded during the reaction. The best fits to the experimental data in Figure 3 were obtained when the reaction rate constants for all possible reactions between hydroxylamine and laccase or oxygen [pathways (a)–(c) in Scheme 1] were set to zero. This suggests very strongly that the regeneration of hydroxylamine to oxoammonium proceeds solely through a combination of comproportionation and laccase-catalyzed TEMPO oxidation, that is, pathway (d) in Scheme 1. In a separate experiment, the rate of oxidation of hydroxylamine by air, as in pathway (a), was determined at $2\ nM\ s^{-1}$ under the experimental assay conditions, which is insufficient to sustain the rate of the measured conversions in Figure 3.[20]

The reaction between benzyl alcohol and oxoammonium in the absence of laccase

The reaction between oxoammonium and benzyl alcohol (Reaction 3) is given by Scheme 4, the reaction rate is given by Equation (3), and the experimental results and fits to the model are presented in Figure 4.



Scheme 4. Reaction 3: Reaction between oxoammonium and benzyl alcohol.

$$\frac{dC_{\text{benzyl alcohol}}}{dt} = -r_3 = -k_3 \times C_{\text{oxoammonium}} \times C_{\text{benzyl alcohol}} \qquad (3)$$

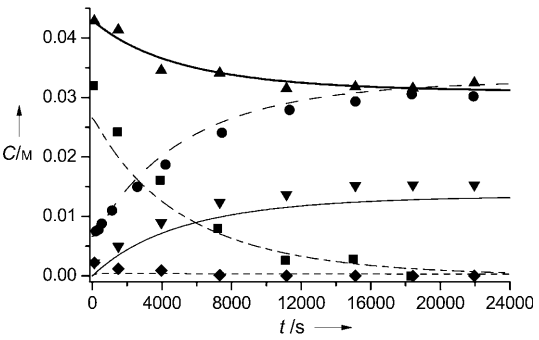


Figure 4. Time course of the oxidation of benzyl alcohol by oxoammonium in the presence of TEMPO. The lines through the data points represent fits based on Reaction 3 (Scheme 4) also including Reaction 2. See text and Table 2 for the fit parameters. Experimental conditions: Initial [benzyl alcohol] = 43.0 mM, [oxoammonium] = 26.5 mM, [TEMPO] = 7.1 mM, $T = 30\ ^\circ C$, pH 4.3, under constant aeration. ● TEMPO (EPR); ◆ hydroxylamine (GC); ■ oxoammonium (IR); ▲ benzyl alcohol (GC); ▼ benzaldehyde (GC); ––– TEMPO (model); ----- hydroxylamine (model); – – – oxoammonium (model); —— benzyl alcohol (model); —— benzaldehyde (model).

The reaction rate constant for this reaction k_3 was found to be $2.2 \times 10^{-3}\ M^{-1}s^{-1}$. The measured stoichiometry of the benzyl alcohol and benzaldehyde are in agreement with Scheme 4.

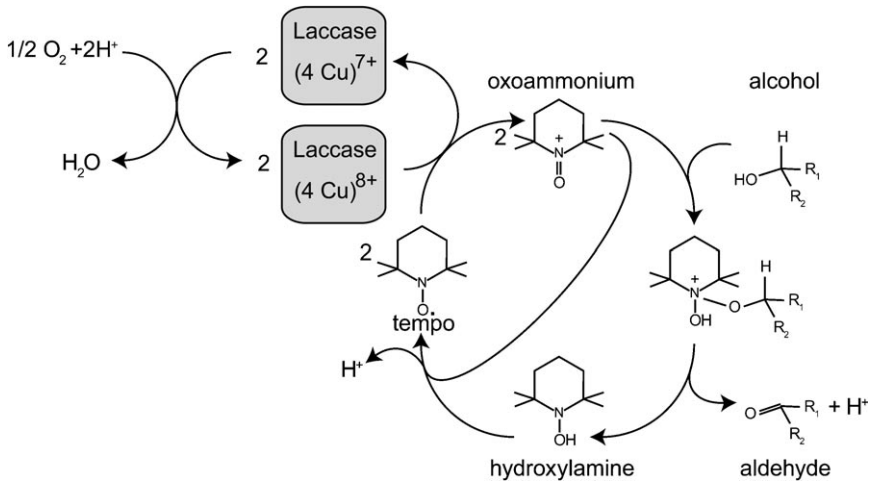
Further analysis by HPLC indicated that no overoxidation to the corresponding benzoic acid had occurred. In addition, the model adequately describes the steady-state concentrations of the hydroxylamine, TEMPO, and the oxoammonium cation. To put the relatively low value for the rate of oxidation of benzyl alcohol by oxoammonium into perspective (we could not find data in the literature for this rate in aqueous solution at values of pH 4–5), a comparison was made with methanol oxidation rates in aqueous buffers (Table 1). The data in Table 1 indicate that the reaction rate we have determined for benzyl alcohol oxidation is close to that for methanol oxidation at similar pH values. Furthermore, the methanol reaction rates are strongly dependent on the pH, which we expect also to be the case for benzyl alcohol.

Table 1. Reaction rate constants for oxidation of methanol and benzyl alcohol by oxoammonium in aqueous buffer at various pH values.

Reaction rate constant [$M^{-1}s^{-1}$]	pH	Alcohol	Reference
3.1×10^{-4}	4	methanol	[21]
2.2×10^{-3}	4.3	benzyl alcohol	this work
2.7×10^{-3}	4.6	methanol	[22]
4.8×10^{-1}	6.9	methanol	[23]

The complete reaction: Conversion of benzyl alcohol by the laccase–TEMPO system

The proposed mechanism for the conversion of benzyl alcohol is shown in Scheme 5. This model includes the three reactions discussed above. The relevant reaction rate constants are listed in Table 2.



Scheme 5. Proposed reaction mechanism of primary alcohol oxidation by oxygen catalyzed by laccase and TEMPO. The mechanism indicated for alcohol oxidation by the oxoammonium cation under experimental conditions is based on the work of Arends et al.[24]

The predicting power of the model was tested in experiments with all of the components present; TEMPO, laccase, and benzyl alcohol (Figure 5). According to the model, laccase is assumed to be in the oxidized state. This assumption was based on the very high reaction rate of ambient oxygen with laccase ($k_{cat} > 1\times10^4\ s^{-1}$).[5] The time courses of all components are estimated adequately by the model except those for benzaldehyde at times longer than 1 h. HPLC analyses indicated a lack of benzoic acid formation as described for the experiment in Figure 4. However, total organic carbon analyses at $t = 60$ s and $t = 17900$ s indicated a loss of 0.17 $mol_{carbon}\,L^{-1}$, corresponding to a loss of 25.3 mM of a seven-carbon compound such as benzaldehyde. At $t = 17900$ s, the measured total combined concentration of benzaldehyde and benzyl alcohol was short by 23.8 mM short (Figure 5). The similarity between these two values indicates that most of the benzaldehyde had evaporated. The difference between the experiments delineated in Figures 4 and 5 is that a significantly more vigorous aeration was

Table 2. Reaction rate constants for oxidation of benzyl alcohol by oxygen by the bicatalytic laccase–TEMPO system.

Reaction	Description	Kinetic parameters
1	oxidation of TEMPO by laccase	$k_1 = 3.6\ s^{-1}$ $K_M = 1.8\times10^{-3}$ M
2	comproportionation reaction	$k_2 = 0.23\ M^{-1}s^{-1}$
3	reaction of oxoammonium and benzyl alcohol	$k_3 = 2.2\times10^{-3}\ M^{-1}s^{-1}$

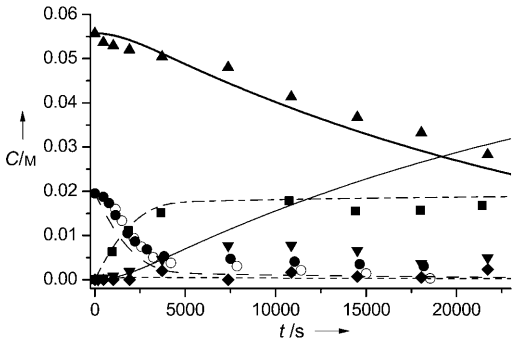


Figure 5. Time course of the oxidation of benzyl alcohol by the laccase–TEMPO system and quantitative analysis of the reaction intermediates and products. The lines through the data points represent fits based on Scheme 5. See text and Table 2 for the fit parameters. Experimental conditions: Initial [TEMPO] = 20.0 mM, [benzyl alcohol] = 56 mM, [laccase] = 2.5 μM. ● TEMPO (EPR); ○ TEMPO (UV/Vis); ◆ hydroxylamine (GC); ■ oxoammonium (IR); ▲ benzyl alcohol (GC); ▼ benzaldehyde (GC); – – – TEMPO (model); ----- hydroxylamine (model); – – – oxoammonium (model); —— benzyl alcohol (model); —— benzaldehyde (model).

applied to that in Figure 5. In this experiment, crucially, oxygen was consumed, and we wanted to ensure that the liquid was saturated with oxygen. This aeration led to evaporation of the benzaldehyde, which was only present as a product (see Scheme 5). The concentration of benzaldehyde is not a variable in the kinetic equations and therefore, stripping it from the liquid had no consequence for the validation of the kinetic scheme. For this reason, we preferred to ensure oxygen saturation rather than preventing benzaldehyde evaporation.

Conclusions

We have elucidated herein the mechanism of benzyl alcohol conversion to benzaldehyde in air, catalyzed by the organocatalyst TEMPO and the enzyme laccase. The quantitative determination of reactant, product, and potential intermediate concentrations by combining EPR spectroscopy, UV/Vis spectroscopy, ATR-FTIR spectroscopy, and GC analysis proved essential in establishing the kinetically significant pathways and the corresponding rate constants. Key observations were the lack of reaction of the hydroxylamine with laccase and the dominant regeneration of the *N*-oxyl species via comproportionation with the oxoammonium cation. The kinetics of alcohol conversion were adequately fit to a model that incorporated these features.

The optimal conversion conditions for primary alcohols, pH 4–5 and 30 °C, appeared to be strongly determined by the general optimum acidic pH of laccases. However, at such low pH values, the reaction between oxoammonium and primary alcohols was slow. The selectivity of alcohol oxidation was determined by the oxoammonium, not by the laccase. Alcohol conversion rates and selectivity of the laccase–TEMPO system might be improved by selection of the best TEMPO derivative in combination with a genetically modified laccase that has a higher pH optimum and that is able to directly oxidize the hydroxylamine of the corresponding TEMPO derivative.

Experimental Section

Materials

Laccase [1.10.3.2] from a strain of *Trametes versicolor* was employed. TEMPO (98%), *n*-hexane, NaH_2PO_4, H_3PO_4, NaAc, HAc, Br_2, DOWEX, ethyl acetate, ether, $MgSO_4$, and anisole were obtained from Aldrich and Acros organics and used as received. The laccase concentration was calculated from the added mass of enzyme, which has a purity of 24% in the commercial powder.[5]

TEMPO was oxidized to the oxoammonium cation by the addition of bromine to a solution of TEMPO in *n*-hexane.[18] The thus-formed oxoammonium bromide was filtered and washed with *n*-hexane and subsequently dried. The oxoammonium bromide was dissolved in water and subjected to a DOWEX 1×8 exchange resin saturated with 1 M NaH_2PO_4–Na_2HPO_4 at pH 4.5. Drying under reduced pressure followed by freeze drying yielded the oxoammonium phosphate salt that was used in the experiments.

To produce hydroxylamine, TEMPO was reduced by ascorbic acid in a minimum amount of water, followed by extraction of the formed hydroxylamine in ether.[19] The ether extract was dried with $MgSO_4$, the solvent was removed by evaporation and the resultant hydroxylamine powder was stored under a nitrogen atmosphere.

Alcohol conversion by the laccase–TEMPO system

Reactions were carried out at 30 °C in a stationary Omni Reaction Station from Electrothermal with aeration by air and temperature control. Air stones were used to ensure sufficient aeration. Control experiments with 100% oxygen instead of air yielded the same results. All reaction mixtures were buffered at pH 4.3 with a 50 mM sodium acetate/acetic acid buffer. Reactions were started by addition of either laccase or oxoammonium from approximately ten-fold-concentrated stock solutions. The total reaction volume was 10 mL. For GC analysis, 200 μL samples were taken and added directly to 1800 μL of ethyl acetate, resulting in a phase separation and quenching of the reaction. Previous experiments[20] showed that oxoammonium and laccase remain in the aqueous phase, whereas TEMPO, benzyl alcohol, and benzaldehyde partition into the ethyl acetate phase. For EPR and UV/Vis spectroscopy, a single 100 μL sample was taken, transferred by pipette into an Eppendorf vial, and directly frozen in liquid nitrogen, effectively quenching the reaction. For FTIR analysis a 100 μL sample was deposited on a diamond ATR crystal and directly measured.

Analytical methods

For GC analysis, 1 mL of the ethyl acetate phase of the GC sample, containing TEMPO, hydroxylamine, benzyl alcohol, and benzaldehyde, was collected and dried over $MgSO_4$. 500 μL of the sample was subsequently added to a GC vial and 200 μL of 10 mM anisole solution in ethyl acetate was added as a standard. GC analysis was carried out with a WAX 52 CB column (50 m×0.53 mm) heated at 80 °C for 5 min followed by temperature increase at 7 °C min^{-1} to 235 °C. To compensate for material losses, the same extraction process was used for the various standards. Due to overlap of the peak areas of TEMPO and hydroxylamine, the concentrations of TEMPO and hydroxylamine could not be resolved and the sum of the two was measured. The hydroxylamine concentration was subsequently calculated by subtracting the TEMPO concentration determined by EPR spectroscopy.

To determine the possible formation of benzoic acid, 50 μL of sample was diluted to 1 mL in 1:4 acetonitrile/water containing 0.1% trifluoracetic acid. The sample was loaded onto a Chromo-Lith Speed Rod RP 18e on an Alliance 2690 HPLC and eluted with 1:9 acetonitrile/water and the same buffer. A Waters 2497 UV detector was used at 210 nm.

To check for possible evaporation of the product benzaldehyde, total organic carbon content was determined on a Shimadzu TOC 5050 A calibrated with potassium hydrogen phthalate. A 50 μL sample of the reaction mixture was diluted in 4 mL of water and subsequently analyzed.

EPR spectroscopy was performed on a Bruker ER200 spectrometer at room temperature in an aqueous sample cell. TEMPO concentrations were determined by double integration of the EPR signal using a 20 mM TEMPO stock solution as a standard. Thawing the 100 μL EPR sample, transferring it by pipette into the aqueous sample cell, and recording the EPR spectrum took approximately 4 min. During the EPR measurement, TEMPO is oxidized by laccase until the solution becomes anaerobic, resulting in an uncertainty of the TEMPO concentration with respect to its true steady-state concentration of 3×10^{-4} M.

UV/Vis spectra were recorded on a HP 8453 UV/Vis spectrometer. After EPR analysis, the sample was transferred by pipette from the aqueous sample cell into a 50 μL cuvette (path-length = 1 cm) and analyzed. The UV/Vis spectrum was recorded approximately 6 min after the EPR spectrum. The concentrations of TEMPO and oxoammonium were determined using a classical least squares (CLS) method in the 380–600 nm range. For this method, the oxoammonium spectrum was given by the oxoammonium formed in the laccase-catalyzed TEMPO oxidation. Baseline correction was implemented by adding a flat baseline and a scatter-correction baseline, given by $A = \lambda^{-4}$, as spectra in the CLS algorithm. After transfer to the cuvette from the aqueous sample cell, the solution becomes (partially) aerobic and the overall reaction may proceed to an unknown extent for approximately 6 min. TEMPO is oxidized by laccase until the solution becomes anaerobic, once again resulting in an uncertainty of the TEMPO concentration with respect to its true steady-state concentration of 1×10^{-3} M.

FTIR samples were analyzed using a Thermo-Nicolet 8700 equipped with a Golden Gate diamond ATR accessory. An in-house-created funnel shaped Teflon sample holder was used to prevent the material surrounding the diamond crystal from interacting with the sample. A resolution of 4 cm^{-1} was used and the final spectrum was averaged from 1024 scans. The oxoammonium concentration was calculated from the baseline-corrected area between 1624 and 1612 cm^{-1} multiplied by 0.018, as found from a linear correlation between known oxoammonium concentrations and absorbance. Water vapor bands, which are dominant at the considered wavenumbers, were subtracted. Remaining noise made quantitative determination of oxoammonium concentrations difficult in some cases.

Simulations

Simulations were performed with MATLAB A2008b, using an ode15s routine for calculation of concentrations at a given time. Reaction rate constants were obtained by minimization of the root mean square difference between experimentally obtained and modeled concentrations, using the lsqcurvefit routine. Kinetic parameters were allowed to be any positive number. Kinetic parameters for Reactions 1 and 2 were determined first and subsequently used for determination of the kinetic parameters for Reaction 3. For comparison of the simulations with the complete bicatalytic cycle, no fitting routines were applied.

Keywords: alcohols · enzyme catalysis · organocatalysis · reaction mechanisms · spectroscopic methods

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