Journal of Inorganic Biochemistry 201 (2019) 110843

Contents lists available at ScienceDirect

Journal of Inorganic Biochemistry

journal homepage: www.elsevier.com/locate/jinorgbio

Correlation between the T1 copper reduction potential and catalytic activity of a small laccase

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ARTICLE INFO

Keywords:
Small laccase
Type 1 (T1) copper
Reduction potential
Axial ligand
Substituted phenols

ABSTRACT

Laccases are multicopper enzymes that catalyze oxidation of electron-rich substrates coupled to reduction of molecular oxygen to water. Since the Type 1 copper (T1 Cu) is the site where electrons are withdrawn from the substrate, it is assumed that the reduction potential of this copper correlates with enzyme activity. Herein, we studied the correlation of the T1 Cu reduction potential and the enzymatic activity of the small two-domain laccase Ssl1 from *Streptomyces sviceus*. For a systematic approach, we aimed to minimize any effects other than the reduction potential difference. To this end, we constructed a series of Ssl1 mutants with reduction potentials varying from < 290 to 560 mV. Along with the hydrophobicity of the axial ligand of the T1 Cu also structural changes in the substrate binding site and additional hydrogen bonding increased the reduction potential. Enzyme activity experiments demonstrated that the T1 Cu reduction potential has a different effect on oxidation of different substrates. Whereas there was no obvious correlation between the T1 Cu reduction potential and kinetic parameters for the oxidation of syringaldazine (with a reduction potential of 390 mV), a good correlation was observed between the T1 Cu reduction potential and the conversion of substituted phenols with reduction potentials between 660 and 820 mV. This correlation was pronounced for the Ssl1 variants with reduction potentials above 470 mV, which demonstrated increased activities also during the oxidation of two dyes, alizarin red S and indigo carmine.

1. Introduction

Laccases (EC 1.10.3.2) are multicopper oxidases that catalyze the one-electron oxidation of substrates coupled with the four-electron reduction of molecular oxygen to water. Known laccase substrates include phenols, aryl amines, anilines, benzenethiols, and metal ions [1–3]. Laccases contain four Cu ions and commonly consist of three cupredoxin-like domains formally numbered 1, 2, and 3. Domain 3 contains one Type 1 (blue) copper site (T1 Cu). Between domains 1 and 3 a trinuclear cluster (TNC) is located that is formed by one Type 2 copper and two Type 3 copper ions [4]. Domain 2 is not involved in the copper binding. The copper sites of laccases are distinguished by their spectroscopic and paramagnetic properties [5]. Along with typical three-domain laccases the so-called small laccases exist that are composed of two domains. These two-domain small laccases form functional homotrimers with the TNC located at the interface of neighboring monomers [6–8].

Although the T1 Cu coordination is similar among the multicopper proteins and involves at least two histidines and one cysteine, the coordination spheres in various enzymes are different [9]. The T1 Cu in plant laccases is coordinated by two histidines, one cysteine, and one methionine as coordinating axial ligand [10]. Fungal laccases possess a non-coordinating residue like leucine or phenylalanine instead of the axial methionine. These differences in coordination geometry were shown to influence electronic structure and electron transfer to the T1 Cu. Among others, the axial ligand of the T1 copper, and particularly presence or absence of the coordinating methionine, is known to determine the T1 Cu reduction potential (E°) [11]. Thus, low-potential laccases with reduction potentials between 340 and 490 mV versus NHE commonly have methionine as the axial ligand of the T1 Cu. Medium-potential laccases have reduction potentials in the range between 470 and 710 mV and non-coordinating leucine in the axial position. Laccases with reduction potentials of 720 to 780 mV are classified as high-potential laccases, which carry in most cases a non-coordinating phenylalanine residue as the axial ligand [12]. Besides metal-ligand interactions, other factors including the hydrophobicity of the residues within 6 Å of the T1 Cu, H-bonding to the coordinating cysteine-sulfur atom, protein constraints, and electrostatic interactions in the protein have been identified as the main determinants of the T1 Cu reduction potential [13–15]. Thus, among laccases with identical ligand sets, E°

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https://doi.org/10.1016/j.jinorgbio.2019.110843
Received 4 June 2019; Received in revised form 22 August 2019; Accepted 7 September 2019
Available online 09 September 2019
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can differ substantially [10].

During laccase-mediated catalysis, four substrate molecules undergo one electron abstractions at the T1 Cu. The electrons abstracted from substrates are subsequently transferred from the T1 Cu to the TNC via the approximately 13 Å long conserved His-Cys-His path - the super-exchange pathway. In the fully reduced form (4 Cu^I) of the enzyme, molecular oxygen is reduced at the TNC [16]. A peroxide intermediate is formed after two two-electron reduction steps and finally water is released [17].

The intermolecular electron transfer from the substrate to the T1 Cu is considered the rate-limiting step of laccase activity [18,19]. According to the Marcus theory the rate constant of the intermolecular electron transfer (k_{ET}) is mainly influenced by the electronic driving force, ΔG° (i.e. the difference in the reduction potential) and the reorganization energy [20]. The reorganization energy is the energy required for substrate and protein rearrangements between initial and final states upon electron transfer [10].

For many substrates catalytic activity of laccases correlates with the difference in half reduction potentials between the T1 Cu and the substrate (ΔE°) [2,21,22]. Laccases can oxidize also the substrates with E° exceeding their T1 Cu E°. The endergonic oxidation of the substrate may be compensated by the exergonic reduction of O_2 to H_2O [23]. Thus, understanding the correlation between the T1 Cu reduction potential and the catalytic activity of laccases has been of great interest [1], and remains a topic of current research [15,24,25]. High-potential laccases have been reported to oxidize recalcitrant dyes and to possess higher activities as compared to middle- and low-potential laccases [23]. Respectively, increasing the T1 Cu E° by protein engineering could not only enhance substrate oxidation rate but also extend laccases' substrate spectra. However, ΔE° does not always clearly correlate with laccase activity. Studies on four laccases from basidiomycetes revealed that for monophenolic substrates with low E°, ΔE° between the T1 Cu and the substrate did not directly influence the laccase activity [24]. Replacement of the axial phenylalanine by methionine in a *Trametes villosa* laccase resulted in an expected decrease of the reduction potential by 110 mV but in increased k_{cat} during the oxidation of phenolic substrates [26]. Obviously, E° is not the only determining factor of laccase activity. Along with the above mentioned reorganization energy, also the electrostatic environment of the substrate [27] and steric hindrances associated with substrates' size [21] affect laccase activity. Furthermore, the substrate binding mode influences the donor-acceptor distance between the substrate donor atom and the T1 Cu; as the electron transfer rate decays exponentially with the donor-acceptor distance it significantly affects k_{cat} [28].

So far, studies on the correlation between laccase activity and the difference in half potentials of the T1 Cu and the substrate (ΔE°) have been conducted mainly on high- and middle-potential fungal laccases and the reported results demonstrate the complexity of this aspect. Since the past decade, bacterial laccases have gained research interest because of their comparably easy heterologous production, lack of glycosylation, activity and stability at alkaline pH and high tolerance towards Cl^- as compared to fungal laccases [29,30]. However, bacterial laccases possess lower activity as compared to fungal laccases. Even though some mutants with changed reduction potential have been reported for bacterial laccases [31–33], the correlation between the T1 Cu reduction potentials of bacterial laccases and their activity remains much less investigated. As small two-domain laccases have been discovered only relatively recently, a respective systematic investigation is still missing.

Thus, the aim of this work was firstly to investigate the factors contributing to the reduction potential of the small two-domain laccase Ssl1 from *Streptomyces sviceus,* and secondly to study the correlation between the T1 Cu reduction potential and the enzyme activity. To this end, we constructed a series of Ssl1 mutants with reduction potentials spanning a range of at least 270 mV and investigated their ability to oxidize several laccase substrates with various sizes and half potentials.

2. Experimental

2.1. Site-directed mutagenesis

Site-directed mutagenesis of *ssl1* was performed using the QuikChange protocol (Stratagene) with primers containing the desired mutation (Table S1) and pET22H_ssl1 or variants thereof as template. Correct sequences of *ssl1* genes were verified by Sanger sequencing (GATC Biotech, Konstanz, Germany).

2.2. Expression and purification of Ssl1

Ssl1 laccase from *S. sviceus* (UniProtKB B5HSR1) and Ssl1 variants were expressed without the natural N-terminal *tat*-signal sequence. Residue numbering follows the sequence containing the natural signal sequence [34]. For expression of *ssl1, E. coli* BL21-CodonPlus(DE3)-RP was transformed with pET22H_ssl1 or variants thereof. Cells were grown in 200 mL terrific broth (TB)-medium in 2 L flasks supplemented with 100 μg/mL ampicillin and 34 μg/mL chloramphenicol at 37 °C and 180 rpm to an OD_{600} of 1.5. Expression was induced with 20 μM isopropyl β-D-1-thiogalactopyranoside (IPTG) and 2 mM $CuSO_4$. After expression for 20 h at 19 °C and 140 rpm, cells were harvested by centrifugation (11,000 x*g*, 30 min, 4 °C), resuspended in 15 mL potassium phosphate buffer (50 mM, pH 7.5) and centrifuged (3082 x*g*, 30 min, 4 °C). Cell pellets were stored at −20 °C.

For lysis cells were resuspended in chilled potassium phosphate buffer (50 mM, pH 7.5) containing 0.3 mM copper(II) sulfate and 0.1 mM phenylmethanesulfonyl fluoride. Cells were lysed by sonication on a Branson Sonifier 250 with three cycles of 60 s (40% duty cycle, output control 4) with at least 60 s incubation on ice between cycles. Cell debris was separated by centrifugation (30,000 ×*g*) and 1.0 mM $CuSO_4$ was added to the soluble fraction. After incubation for 2 h at room temperature or until no further increase in activity was observed, the lysate was subjected to a heat precipitation (20 min, 65 °C). Precipitated proteins were removed by centrifugation (50,000 ×*g*, 30 min, 4 °C).

After heat precipitation of most endogenous *E. coli* proteins, further purification of Ssl1 was achieved by immobilized metal ion affinity chromatography (IMAC) using a HiTrap TALON crude 5 mL column (GE Healthcare, München) on an ÄKTApurifier 100 fast performance liquid chromatography (FPLC) system (GE Healthcare, München). The column was equilibrated with five column volumes (CV) binding buffer (50 mM potassium phosphate buffer, 500 mM sodium chloride, pH 7.5) and the filtered (0.45 μm) supernatant from the heat precipitation was applied with 1 mL/min. The column was washed with two CV binding buffer, five CV washing buffer (5 mM imidazole) and Ssl1 was eluted with five CV of elution buffer (150 mM imidazole). The elution of proteins was monitored by absorption at 280, 330, and 600 nm. Ssl1 containing fractions were pooled, concentrated (Vivaspin Turbo 15, MWCO of 10 kDa, Sartorius, Göttingen) and the elution buffer was replaced by 50 mM potassium phosphate buffer (pH 7.5) using PD miditrap G-25 columns (GE Healthcare, München). Protein concentrations were determined using the Bradford method [35].

2.3. Determination of reduction potentials

The reduction potentials were determined by redox titrations with the redox couple potassium hexacyanoferrate(III)/potassium hexacyanidoferrate(II) ($E'^\circ = 0.433$ V) as mediator under nitrogen atmosphere. Reduced Ssl1 (50 μM) in 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES, pH 7.0) and 10 mM potassium hexacyanidoferrate(II) was gradually oxidized by adding a solution containing 50 μM Ssl1 and potassium hexacyanoferrate(III) (10–900 mM) in 10 mM HEPES (pH 7.0). Oxidation of the T1 Cu was followed spectrophotometrically on a TIDAS E photo diode array spectrophotometer (J&M Analytik, Essingen, Germany) using the increase in absorption at ~600 nm (Figs. S4, S5). After each step, a spectrum from 340 to 1000 nm was recorded. Reduction potentials were calculated according to the Nernst equation. The absorption at ~600 nm (y) was plotted against the reduction potential (x) and fitted to the Nernst equation $y = A_2 + \frac{A_1 - A_2}{1 + \exp\left(\frac{x - x_0}{dx}\right)}$ $with\ dx = \frac{RT}{nF} = 0.02526\ J/C$ for one electron oxidations/reductions) in OriginPro 9G (OriginLab Corporation, Northampton, MA, USA). The fit parameter x_0 represents the midpoint potential of the laccase (Figs. S6-S13).

2.4. Determination of catalytic constants for syringaldazine conversion

Kinetic constants of Ssl1 variants were determined with the typical laccase substrate syringaldazine (SGZ). Reactions were performed at 25 °C in 50 mM potassium phosphate buffer (pH 8.0) and 10% (v/v) dimethyl sulfoxide (DMSO) for substrate solubilization. SGZ concentrations of 0.4–400 μM were used. Product formation was followed spectrophotometrically at 525 nm (65 mM-^{1} cm^{-1}) on a Tecan Infinite PRO 200 (Tecan, Männedorf, Switzerland) reader. Initial reaction rates were determined in triplicate according to the Lambert-Beer law and were fitted by non-linear regression to the Michaelis-Menten equation $v = v_{max}[S]/(K_M + [S])$ in OriginPro 9G (Fig. S3). k_{cat} values were calculated based on the protein concentration determined by the Bradford method.

2.5. Decolorization of dyes

The decolorization of alizarin red S and indigo carmine by Ssl1 variants was analyzed as previously described [7]. Conversion rates were determined by comparing the residual absorption at 608 nm for indigo carmine and 513 nm for alizarin red S, respectively, with the absorption of the control reactions without enzyme.

2.6. Conversion of substituted phenols

Phenol oxidation reactions contained 1 mM substrate, 1 μM enzyme, and 1% (v/v) ethanol for substrate solubilization in 1 mL potassium phosphate buffer (50 mM, pH 8.0). After incubation for 24 h at 25 °C with overhead rotation (15 rpm) reactions were stopped by addition of 30 μL 6 M hydrochloric acid. Denatured protein was separated by centrifugation (12.300 g, 5 min). The reactions were analyzed using high-performance liquid chromatography (HPLC) on a Shimadzu HPLC system (Shimadzu, Duisburg, Germany) equipped with a Chromolith Performance RP-18e column (100 × 4.6 mm, Merck, Darmstadt, Germany). The injection volume was 5 μL. A solvent gradient of acetonitrile and 0.1% formic acid was applied at a flow rate of 1 mL/min. The acetonitrile concentration was increased from 5 to 100% in 10 min, held at 100% for 3 min, followed by re-equilibration of the column with 5% acetonitrile for 4 min. A photodiode array was used for detection of compounds by monitoring absorption between 190 and 800 nm. Remaining substrate concentrations were calculated from the substrate peak areas at 280 nm using calibrations curves determined from 50 to 1000 μM. The substrate concentration in control reactions after 24 h was used as reference for calculation of conversion rates.

2.7. Calculation of ClogP

ClogP values were calculated as described by Garner et al. [36] using the Bio-Loom program available at http://www.biobyte.com/bb/prod/bioloom.html that is based on the fragment method by Abraham and Leo [37].

3. Results

3.1. Correlation between the axial ligand hydrophobicity and the reduction potential

In our previous work on the two-domain laccase Ssl1 from *S. sviceus* we demonstrated that replacement of the axial T1 Cu ligand methionine by leucine (M295L) increased *E*° [7]. In the present study, we tested the effect of the axial ligand by introducing not only phenylalanine, the third naturally occurring axial ligand in laccases, but also tyrosine, valine, isoleucine, alanine, and threonine. Substitution of the axial methionine by alanine in *Alcaligenes xylosoxidans* nitrite reductase increased *E*° from 240 to 314 mV [38,39]. Threonine was chosen, because the exchange of the axial methionine with threonine in *Rhodobacter sphaeroides* nitrite reductase led to great changes in the electronic structure [40]. Valine, isoleucine, and tyrosine were identified as interesting candidates for axial ligands with increased *E*° due to their similarities to leucine and phenylalanine, respectively.

All mutants were expressed in *E. coli* and purified to homogeneity yielding up to 300 mg Ssl1 per liter of culture and their Cu loading was analyzed (see Supporting Information). The correlation between reduction potential and the side chain hydrophobicity of the axial ligand was linear with an R^2 of 0.84 (Fig. 1) with the highest potentials for M295I and M295L. The potential decreased in the row M295F, M295V, M295Y and WT with methionine 295. The reduction potentials of M295A and M295T could not be determined using the ferro/ferricyanide redox couple. They were lower than 290 mV. These results confirm the axial ligand hydrophobicity as an essential contribution to the increase of *E*°.

3.2. Combination of mutations can further increase reduction potential

However, the axial ligand hydrophobicity is not the only factor influencing the T1 Cu reduction potential. We therefore sought to examine, if the reduction potential of Ssl1 can further be increased by introducing additional mutations in the second sphere of the T1 Cu. In our previous study we found that mutation of M195 and M293 (both located in the putative substrate binding site and in close proximity to the T1 Cu) influenced the reduction potential of Ssl1 (Fig. 2A) [7]. Starting from Ssl1 M295L (456 mV) we first added the M293G mutation and thereby another 40 mV to the T1 Cu reduction potential (Fig. 3).

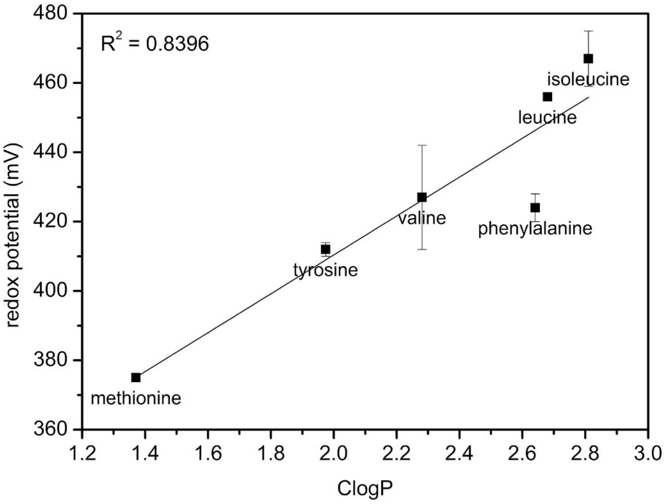

Fig. 1. Correlation of the T1 Cu reduction potential and the ClogP as measure of hydrophobicity of the amino acid residue at the axial position. Values for wildtype (methionine) and M295L (leucine) are from Gunne et al. [7]. The correlation can be described with the following equation: $E° = 298 + 56 \times$ ClogP. Error bars represent SD of triplicates.

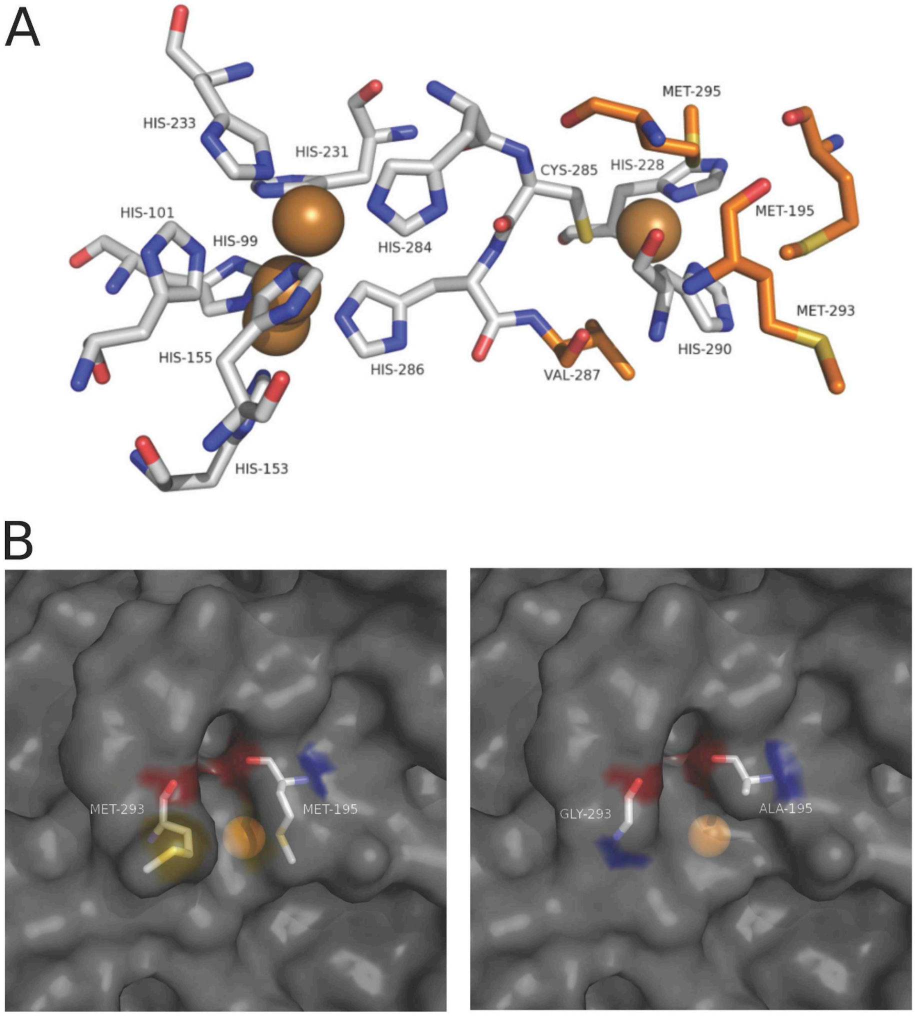

Fig. 2. A) Structure of the Ssl1 active site (PDB 4M3H). Copper ions are depicted as spheres. Positions subjected to mutagenesis are highlighted in orange. B) Location of the M195A and M293G mutations in the putative substrate binding site of Ssl1. The orange sphere represents the T1 Cu ion. Mutations were modeled using the PyMol mutagenesis tool.

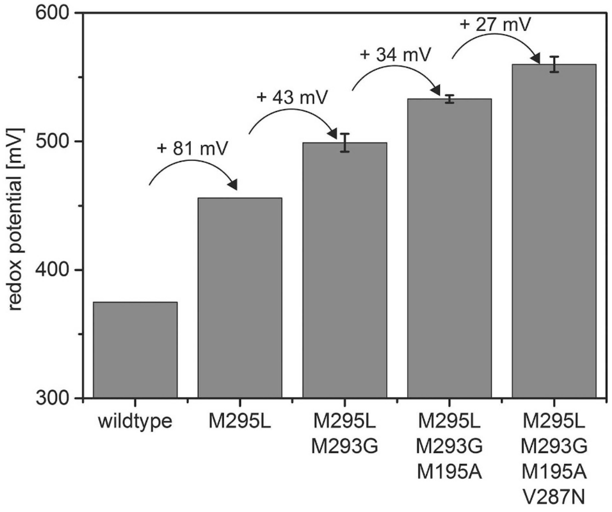

Fig. 3. Increasing T1 Cu reduction potential in Ssl1 mutants. Error bars represent SD of triplicates. Values for wildtype and M295 L are from Gunne et al. [7].

The M195A substitution contributed another 30 mV. Both the M195A and M293G mutations should enlarge the putative substrate binding site (Fig. 2B) as previously described for another small laccase SLAC [41].

Electron density near the T1 Cu favors Cu^{II} over Cu^{I} through ionic interactions, thereby lowering $E°$ [14,42]. In Ssl1 the carbonyl oxygen of Y227 is located 4.1 Å away from the T1 Cu (see Fig. S1), possibly contributing electron density to the T1 Cu site. The protein backbone cannot be addressed directly through mutations, but introduction of a hydrogen bond donor in the carbonyl-O vicinity can redirect electron density. Based on the previous mutational studies on azurin [42] and the two-domain small laccase from *Streptomyces coelicolor* (SLAC) [33] we identified V287 as a suitable residue for hydrogen bond formation with the carbonyl-O of Tyr227 and introduced asparagine (Fig. 2A and Fig. S1). The T1 Cu reduction potential in Ssl1 V287N increased by 139 mV to 514 mV, the highest potential measured for single mutants of Ssl1 so far. The Ssl1 reduction potential was increased by another 30 mV after adding the V287N mutation to the triple mutant. The quadruple Ssl1 mutant M195A/V287N/M293G/M295L has a T1 Cu reduction potential of 560 mV, making it a medium-potential laccase (Fig. 3).

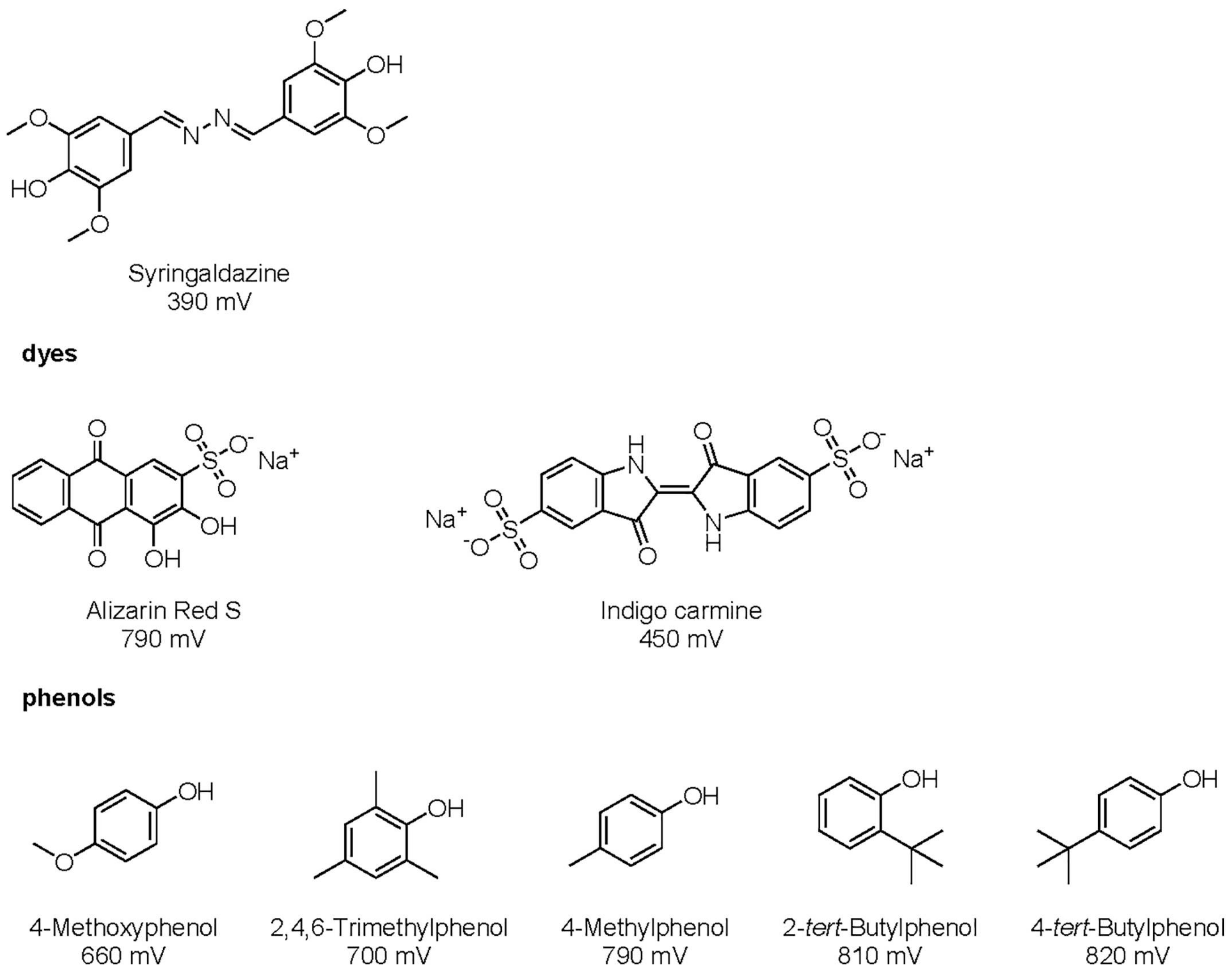

Fig. 4. Tested laccase substrates and the reduction potentials for syringaldazine [43], alizarin red S [45], indigo carmine [46] and phenols [21].

3.3. Activity of Ssl1 variants towards different substrates

For evaluation of the effect of the T1 Cu reduction potential on enzyme activity we tested the typical laccase substrate syringaldazine with a reduction potential of 390 mV at pH 7 [1,43,44], high-potential substrates like the dyes alizarin red S [45] and indigo carmine [46] as well as substituted phenols [21] (Fig. 4). Kinetic constants for syringaldazine oxidation correlated with the difference between the half potentials of T1 Cu and substrate (ΔE°) only partially (Table 1 and Fig. S2). The two mutants M295A and M295T with lower E° as compared to the wild type indeed displayed decreased k_{cat} and increased K_M values and therefore a 100–600-fold decreased catalytic efficiency. The amino acid substitutions M295L, M293G, M195A alone had no or only small effect on K_M. But when included in double or triple mutants, resulted in decreased K_M values which suggests altered substrate binding modes (Table 1).

Although the k_{cat} values decreased for all mutants, two mutants were able to oxidize syringaldazine with increased catalytic efficiency due to lower K_M, namely V287N (1233 mM^{-1} min^{-1}) and M195A/M293G/M295L (2000 mM^{-1} min^{-1}). Surprisingly, the quadruple mutant M195A/V287N/M293G/M295L demonstrated a very low activity, probably due to incomplete copper loading with only 2 Cu per monomer (Table S2). Copper content determination using the bicinchoninic acid method (see Supplemental Information) only gives an average value for the Cu content and does not provide any information regarding the distribution of Cu ions and the amount of fully Cu loaded and active enzyme in the sample. Changes in the oxidative activity of this mutant cannot be directly attributed to the effects of mutations and the increased reduction potential and we therefore excluded this mutant from further experiments.

Further, the activity tests were performed with the dyes alizarin red S and indigo carmine, which are more difficult to oxidize [7,24]. Our results demonstrated that despite their increasing effect on E°, the mutations M295Y, M295V, and M295F impaired decolorization (Fig. 5). The effect was more pronounced for alizarin red S, where conversion decreased from 55% (with WT) to 10–20%. Variants with isoleucine and leucine in the axial position (M295I, M295L) converted both dyes similarly to the wildtype enzyme. This is in accordance with the previously reported values for wildtype and M295L [7]. For mutants with T1 Cu reduction potential of at least 499 mV, higher activities were observed with both dyes. Out of the mutants with lower E° than that of wild type, only Ssl1 M295A (< 290 mV) was able to convert indigo carmine (6% in 24 h) but not alizarin red S. Generally, for dye decolorization substitutions enlarging the putative substrate binding site of Ssl1 were more beneficial (Fig. 5).

For a more systematical analysis of the correlation between ΔE° and laccase activity we searched for compounds differing in their potentials but with only small structural differences. Five substituted phenols with

Table 1
Kinetic constants for syringaldazine conversion and T1 Cu redox potentials of Ssl1 variants. Values are given as means of triplicates ± SD.

	Kinetic constants for syringaldazine			Redox potential
	K_M [μM]	k_{cat} [min^{-1}]	k_{cat}/K_M [$mM^{-1} min^{-1}$]	[mV]
M295A	100 ± 2.2	0.1 ± 0.03	1	< 290
M295T	52 ± 4.1	0.3 ± 0.07	6	< 290
wildtype	30 ± 2.7	19 ± 2.5	633	375 [7]
M293G	43 ± 8.5	13 ± 2.3	302	387 ± 6
M195A	19 ± 4.4	16 ± 1.6	842	408 ± 3
M295Y	190 ± 53.0	3.7 ± 1.0	20	412 ± 2
M295F	37 ± 2.2	4.9 ± 0.5	132	424 ± 4
M295V	80 ± 3.8	7.6 ± 1.7	95	427 ± 15
M295L	27 ± 1.9	13 ± 1.1	481	456 [7]
M295I	45 ± 8.8	7.5 ± 1.5	167	467 ± 8
M293G/M295L	9.0 ± 0.5	2.8 ± 0.3	278	499 ± 7
V287N	3.0 ± 0.1	3.7 ± 0.5	1233	514 ± 7
M195A/M293G/M295L	3.7 ± 0.2	7.4 ± 1.5	2000	533 ± 3
M195A/V287N/M293G/M295L	7.5 ± 1.1	0.004 ± 0.001	0.533	560 ± 6

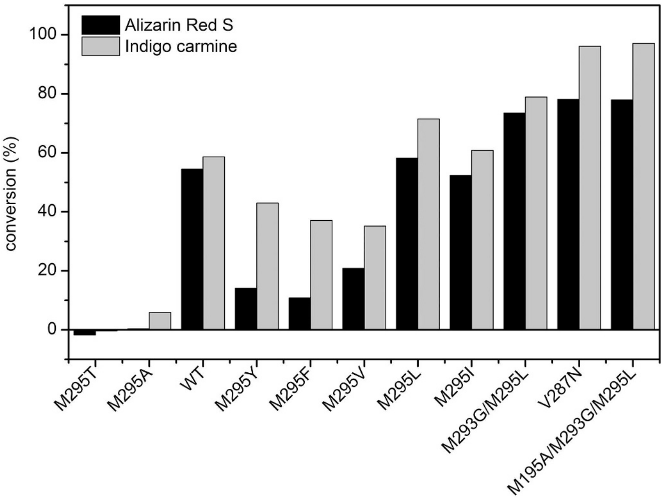

Fig. 5. Conversion of alizarin red S after 4 h (black columns) and indigo carmine after 24 h (grey columns) by Ssl1 wildtype (WT) and variants. Conversion rates are given as means of triplicates. The standard deviation was always < 1.1%.

E° ranging from 660 to 820 mV (Fig. 4) were then tested with the Ssl1 variants.

Although the exact reduction potential of the M295A and M295T mutants could not be determined so far, for ease of comparison these mutants were included in the analysis using a $E^\circ = 290$ mV, because it is the highest possible reduction potential for these variants (Fig. 6 and Fig. S14). Ssl1 M295A and Ssl1 M295T only converted 8 and 5% of 4-methoxyphenol (660 mV) in 24 h but no further substituted phenols. With increasing T1 Cu E°, the conversion rates generally increased and with increasing substrate E° they decreased. While conversions of 4-methoxyphenol (660 mV) were between 59 and 99%, the maximum conversion for 4-*tert*-butylphenol (820 mV) was 29%. For variants with $E^\circ < 500$ mV conversion of 4-*tert*-butylphenol was < 15% (Fig. S14).

It is worth to emphasize that the half potentials of all substituted phenols were higher than the half potentials of all Ssl1 mutants, so that ΔE° in Fig. 6 is negative. Generally, the correlation between ΔE° and laccases' activities has a sigmoidal shape with an inflection point at approximately −275 mV (Fig. 6). A detailed analysis reveals that substituted phenols with reduction potential of < 700 mV were oxidized by all Ssl1 mutants (except for the M295A and M295T with the lowest reduction potential) and the phenols with reduction potentials of ≥790 mV were oxidized by the Ssl1 variants with reduction potential of ≥456 mV.

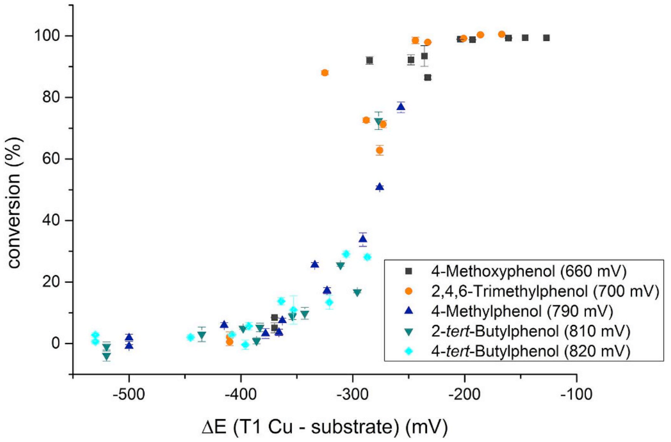

Fig. 6. Correlation between conversion of phenolic compounds and ΔE°. Conversion of 4-methoxyphenol (grey square), 2,4,6,-trimethylphenol (orange dot), 4-methylphenol (dark blue triangle), 2-*tert*-butylphenol (cyan triangle), and 4-*tert*-butylphenol (light blue diamond) by laccase variants with different E° was determined after 24 h. Error bars represent SD. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

4. Discussion

4.1. Redox potentials

Replacement of the axial coordinating methionine by a non-coordinating residue like leucine or phenylalanine has been identified as a factor significantly influencing reduction potentials of multicopper oxidases [10]. Further, the hydrophobicity of the axial ligand has been described as the major determinant of the reduction potential for several T1 Cu containing proteins, including azurin, three-domain laccase, nitrite reductase, rusticyanin, and stellacyanin [36]. In our previous work we demonstrated that the T1 Cu reduction potential increased by 81 mV through mutation of the axial methionine to leucine in the small laccase Ssl1 [7]. We now extended our studies towards axial ligands other than the wild-type methionine and leucine. The observed linear correlation between E° and ClogP value confirmed that the hydrophobicity of a non-coordinating axial ligand is the dominant factor for the reduction potential changes also in two-domain laccases. Extrapolation of the reduction potentials for Ssl1 M295A and M295T from the ClogP values (alanine: 1.103; threonine: −0.235) gives theoretical E° values of 360 mV and 285 mV, respectively. That the reduction potential of the M295A mutant is in fact lower than the theoretical value indicates that the amino acid hydrophobicity is not the only contribution to the reduction potential decrease in this case. The axial position of the T1 Cu might become accessible to a water molecule when residues with smaller side chains are introduced. Based on spectroscopic analysis, presence of a water molecule in the axial position was proposed for the M510A and M510T mutants of the cuprous oxidase CueO from *E. coli* [32]. These two mutants also possess lower reduction potentials as compared to the wildtype CueO.

Even though phenylalanine is the axial ligand of T1 Cu sites in high-potential fungal laccases, E° of the Ssl1 M295F mutant was lower (424 mV) than of the M295L mutant (456 mV). Similar effects were observed in the three-domain laccase CotA from *Bacillus subtilis* when the axial methionine was mutated to leucine or phenylalanine [31]. The M502L mutation increased the T1 Cu reduction potential of CotA from *B. subtilis* by 93 mV (and by 81 mV induced through the homologous mutation in Ssl1), whereas the introduction of phenylalanine led to a smaller reduction potential increase (60 mV in CotA, 49 mV in Ssl1).

Decreasing *E*° by reverse mutations in fungal laccases has also been described. In the *T. villosa* laccase, the wildtype with phenylalanine as axial ligand had the highest *E*° (790 mV). Mutation to leucine decreased the ruduction potential by 50 mV, and to methionine by 110 mV. Mutation of the leucine to methionine in the high-potential *B. aclada* laccase (720 mV) lowered the T1 Cu reduction potential to 580 mV [47]. In *M. thermophila* and *R. solani* laccases mutation of leucine to phenylalanine had no impact on *E*° at all [48].

The reduction potential of the Ssl1 variants could further be increased by adding mutations in the second sphere of the T1 Cu, however the observed effect was generally weaker (Fig. 3). By replacing M195 and M293 by a smaller residue like alanine and glycine for enlarging the substrate binding site (Fig. 2B) in the Ssl1 M295L mutant, we achieved further 30–40 mV increases of *E*°. In contrast to the M195L and M293L mutations, that we previously described [7], this enhancement cannot be attributed to the increased hydrophobicity in the second sphere, because alanine and glycine are less hydrophobic than methionine based on their side chain ClogP values (1.103 and − 1.316, respectively). These alterations might be due to the structural changes of the protein backbone that influence the T1 Cu site. Confirmation of this hypothesis requires structural information from crystallization experiments, which are in progress. By introducing the additional mutation V287N (+27 mV) we created a quadruple mutant with a reduction potential of 560 mV. Our results demonstrate that the M195A, M293G, V287N, and M295L mutations affected the reduction potential not independently, because their individual effects were not additive in the multiple mutants. For example, the M293G mutation led to a +12 mV increase in the wildtype Ssl1 and a +43 mV increase in the M295L mutant. In contrast, the M195A mutation had the same effect in various multiple mutants. The M195A single mutant has a reduction potential of 408 mV (+33 mV compared to the wildtype) and the difference between the M293G/M295L double and the M195A/M293G/M295L triple mutant was +34 mV. Introduction of the fourth mutation V287N to this triple mutant increased the potential by only 27 mV, whereas it led to the largest increase observed for a single mutant (+139 mV compared to the wildtype). Differently, in azurin individual effects of three mutations influencing different aspects of the T1 Cu were additive [42]. However, effects observed in blue copper proteins like azurin containing only one reduction active copper ion are not directly transferrable to multicopper proteins like laccases, where other factors like the presence of the trinuclear cluster also play a role [49]. Possibly, the +139 mV increase in reduction potential of Ssl1 achieved through insertion of asparagine instead of valine 287 might act via the redirection of electron density from a backbone carbonyl through hydrogen bond formation as was described for the azurin mutant F114N (+129 mV) (Fig. S1) [42].

4.2. Laccase activity

Increasing reduction potential and thereby laccase activity has long been of research interest, and the correlation between laccase activity and the reduction potential of the electron accepting copper ion in the active site has been intensively studied [1,21,32,50].

In our study, the Ssl1 activity only partly correlated with the T1 Cu reduction potentials (Fig. S2). For syringaldazine oxidation, replacement of the axial methionine resulted in reduced k_{cat} values. Decrease in k_{cat} was more pronounced for the phenylalanine mutant as shown also for the laccase CotA from *B. subtilis* [31]. However, along with T1 Cu reduction potential, other factors have been described to influence laccase activity and should also be considered during protein engineering for higher laccase activity [51,52]. In some studies, the electronic coupling and the reorganization energy associated with electron transfer were suggested to have a stronger effect on the catalytic activity than Δ*E*° between the T1 Cu and the substrate [26]. For instance, the M298F mutation of the axial ligand in the SLAC laccase from *S. coelicolor* resulted in increased reduction potential but decreased oxidation activity towards 2,2'-azino-bis(3-ethylbenzothia-zoline-6-sulphonic acid (ABTS) and 2,6-dimethoxyphenol (2,6-DMP) [33]. A similar effect was described for the F463M mutant of *T. villosa* laccase with decreased T1 Cu reduction potential which unexpectedly demonstrated increased oxidative activity as compared to the wild type. This was explained by a stronger effect of the changed electronic coupling and/or the reorganization energy as compared to a decreased thermodynamic driving force (ΔG°) and thus a lower kET [26]. The effect of electronic coupling and reorganization energy associated with electron transfer in Ssl1 should be elucidated in further spectroscopic studies of Ssl1 variants.

Often substrate binding event has a greater effect on activity than the reduction potential, as shown for *M. thermophila* laccase (460 mV) and *P. cinnabarinus* laccase (790 mV) in computational studies [53]. K_M values have been shown to correlate with the affinity of the substrates for the T1 binding pocket. Christensen and Kepp developed a model in which active poses should represent short donor-acceptor distances from substrate donor atom to T1 Cu [28]. In our study in the reaction with syringaldazine the amino acid substitutions M295L, M293G, M195A had a small effect on K_M, but when combined in the triple mutant (with *E*° of > 500 mV), resulted in a 3–10-fold decreased K_M values and as consequence in increased catalytic efficiencies (Table 1). These strong alterations might result from different syringaldazine binding modes, due to the binding pocket enlarging mutations M195A and M293G. Another effect to be considered for the mutants containing smaller residues in the substrate binding pocket of Ssl1 (M195A and M293G) is a decreased hydrophobic interaction with the substrate as observed in *T. versicolor* laccase mutants F265A and F332A [54].

For oxidation of alizarin red S and indigo carmine we observed the influence of several factors on laccase activity as well. Whereas alizarin red S has a higher potential of 790 mV, indigo carmine with a potential of 450 mV is bulkier and less flexible. Conversions of indigo carmine were unexpectedly lower than those of alizarin red S, which confirms the observation made with other laccases that not only redox features of the substrate (contributing to potential differences), but also substrate structure is important for the effective oxidation by laccase [21]. Apart from steric hindrance that diminishes laccase activity, the substrate geometry that influences the donor−acceptor distance, and the reorganization energy influence laccase activity [55]. In case of alizarin red S, Δ*E*° is negative for all Ssl1 variants. Our results indicate that the oxidation of alizarin red S may be endergonic by 300–400 mV, because this substrate (790 mV) was oxidized (though with a rather low efficiency) by the wild type Ssl1 (375 mV) and all variants with higher *E*° (Fig. 5). As activity profiles for alizarin red S and indigo carmine oxidations are similar, and the Ssl1 variants with the enlarged putative binding site were more active (Fig. 5), the steric hindrance issue cannot be excluded for alizarin red S as well.

For the substituted phenols indeed a correlation between activity and *E*° of the Ssl1 laccases was observed. Similarly to alizarin Red S, Δ*E*° is negative for all Ssl1 variants and the tested phenolic compounds. Our results (Fig. 6) indicate that the oxidation of substituted phenols may be endergonic by no more than 300 mV, similar to previous observations by Tadesse et al., who investigated *M. thermophila* laccase (460 mV) and *T. villosa* laccase (790 mV) [21]. They also found a correlation between the conversion of substituted phenols and the laccase reduction potential. While *T. villosa* laccase was able to oxidize substrates with E° up to 1.2 V, *M. thermophila* laccase only oxidized substrates with $E^\circ < 800$ mV. For the phenolic compounds we expected the same or at least a very similar binding modes, resulting in similar donor-acceptor distances, and therefore ΔE° as the major factor contributing to the enzyme activity. However, the Ssl1 variants with the highest E° ($\geq$ 514 mV) did not convert 4-methylphenol, 2-*tert*-butylphenol and 4-*tert*-butylphenol (with very similar E° values of 790, 810, and 820 mV, respectively) to the same extent (Fig. S14). The decreased activity towards 2-*tert*-butylphenol and 4-*tert*-butylphenol can be explained by the fact that although *tert*-butyl is electron donating, this bulky alkyl group has a strong steric effect which obviously influences substrate affinity and thus laccase activity.

In summary, in this study we demonstrated that that hydrophobicity of the axial ligand is the major determinant for the reduction potential changes observed in mutants of the small two-domain laccase Ssl1 from *S. sviceus*. By combining substitutions of the axial ligand with mutations in the second sphere of the T1 Cu we developed a middle-potential bacterial laccase with a reduction potential of 560 mV. The Ssl1 variants with $E^\circ \geq 500$ mV enabled oxidation of 4-methylphenol, 2-*tert*-butylphenol and 4-*tert*-butylphenol, which were not accepted as substrates by the wild type enzyme (375 mV). Laccase activity towards substituted phenols correlated with the difference in reduction potentials of the substrates and T1 Cu. For the dyes alizarin red S and indigo carmine such correlation was observed for the Ssl1 variants with $E^\circ > 450$ mV. Our results demonstrate that the difference in reduction potentials of the substrate and the T1 Cu is not the only factor that influence the activity of Ssl1 variants. For indigo carmine, alizarin red S and the *tert*-butyl substituted phenols steric hindrance/substrate affinity have a strong effect on laccase activity.

Abbreviations

CV	column volume
FPLC	fast performance liquid chromatography
HPLC	high performance liquid chromatography
IMAC	immobilized metal ion affinity chromatography
IPTG	isopropyl β-D-1-thiogalactopyranoside
SGZ	syringaldazine
T1 Cu	Type 1 copper
T2 Cu	Type 2 copper
T3 Cu	Type 3 copper
TNC	trinuclear cluster

Declaration of competing interest

The authors declare no competing interest.

Acknowledgments

We thank the Heinrich Heine University Düsseldorf for financial support.

Funding

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jinorgbio.2019.110843.

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