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Characterization of SLAC: A small laccase from *Streptomyces coelicolor* with unprecedented activity

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Abstract

Laccases and other four-copper oxidases are usually constructed of three domains: Domains one and three house the copper sites, and the second domain often helps form a substrate-binding cleft. In contrast to this arrangement, the genome of *Streptomyces coelicolor* was found to encode a small, four-copper oxidase that lacks the second domain. This protein is representative of a new family of enzymes—the two-domain laccases. Disruption of the corresponding gene abrogates laccase activity in the growth media. We have recombinantly expressed this enzyme, called SLAC, in *Escherichia coli* and characterized it. The enzyme binds four copper ions/monomer, and UV-visible absorption and EPR measurements confirm that the conserved type 1 copper site and trinuclear cluster are intact. We also report the first known paramagnetic NMR spectrum for the trinuclear copper cluster of a protein from the laccase family. The enzyme is highly stable, retaining activity as a dimer in denaturing gels after boiling and SDS treatment. The activity of the enzyme against 2,6-dimethoxyphenol (DMP) peaks at an unprecedentedly high pH (9.4), whereas the activity against ferrocyanide decreases with pH. SLAC binds negatively charged substrates more tightly than positively charged or uncharged molecules.

Keywords: laccase; NMR; activity; multicopper; oxidase; copper; PPO

The laccase class of enzymes is widely distributed throughout nature and exhibits a wide range of roles and substrate specificity. They are sometimes also referred to as polyphenol oxidases (PPOs) and are a part of the multi-copper oxidase family (Messerschmidt 1997). The first laccase was discovered in the tree *Rhus vernicifera* more than a hundred years ago (Yoshida 1883). The plant and fungal laccases are similar in that they have low substrate specificity, reacting with a host of derivatized phenols with K_m of a few millimolar. The lack of substrate specificity often leaves the natural role of laccases in question, but this versatility has allowed new roles to be found for many in several commercial processes, including delignification, decolorization of industrial waste, and wine clarification (Mayer and Staples 2002).

In contrast, ceruloplasmin (from the plasma of vertebrates) and Fet3p (from *Saccharomyces cerevisiae*) bind

and oxidize Fe(II) specifically. CueO and PcoA are involved with metal homeostasis in *Escherichia coli* (Rensing and Grass 2003). Ascorbate oxidase, found in many plants, uses ascorbate as a substrate, but for unknown purposes. In this article, all of these proteins will be denoted laccases.

With the recent advent of available genomic data, these enzymes are being isolated from a wide range of organisms, with a large increase in the number of enzymes originating from prokaryotic sources (Claus 2003). Highly conserved features of these proteins include a copper ion bound in a type 1, or blue, site and three more copper ions bound in a trinuclear cluster that is sometimes described as a sum of type 2 and type 3 sites. The polypeptide architecture typically consists of three cupredoxin domains: Disulfide bonds link domain one with domains two and three, and the trinuclear cluster bridges the first and third domains. The reducing substrate is bound in a cleft at the surface and is oxidized by the nearby type 1 site in domain three. Electrons donated by the reducing substrate are transferred to the trinuclear cluster, where dioxygen, the cosubstrate, is reduced to two water molecules (Solomon et al. 1996). Two histidine ligands for the cluster and the cysteine ligand for the

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type 1 site are consecutive amino acids in sequence (HCH), and thus the distance between the type 1 site and the trinuclear cluster as measured by the number of intervening covalent bonds is quite small.

The substrate-binding clefts of laccases are normally constructed from one loop at the end of domain one, two β -turns from domain three, and several residues from the second domain. A recent crystal structure of the *Melanocarpus albomyces* laccase (MaL; Hakulinen et al. 2002) implicates several extended loops in domain two that help form a more confining binding pocket, whereas a structure of *Trametes versicolor* laccase (TvL; Piontek et al. 2002) shows a phenylalanine from domain two in proximity to the substrate. Messerschmidt and coworkers (Messerschmidt et al. 1992) proposed that two tryptophans and a histidine from the second domain in zucchini ascorbate oxidase stabilize the lactone ring of the substrate, ascorbate. Defining the structural constraints for substrate binding is important for applications of these enzymes.

Analysis has suggested that the laccases are the result of replicating a two-domain motif (Messerschmidt and Huber 1990), both domains having cupredoxin folds. Murphy et al. (1997) have predicted that such a precursor could not function as a monomer of two domains. EpoA, a two-domain laccase, has been recently found in *Streptomyces griseus* but operates as a trimer (Endo et al. 2002). This trimer is stable and active on SDS-PAGE gels, similar to a tetrameric laccase from *Gaeumannomyces graminis* var. *tritici* found earlier (Edens et al. 1999).

An important parameter for laccases is the pH-dependence of their activity. For phenolic substrates, the pH-dependence is bell-shaped: An increase with pH is attributed to the pH-dependent decrease in reduction potential of the phenols that formally can be connected to the phenol-phenolate interconversion, whereas the competing atrophy of activity with pH has been ascribed to hydroxide inhibition at the trinuclear cluster (Xu 1997). The maximum of this bell-shaped curve occurs at acidic pHs but has been found as high as pH 9.0 for one laccase. Activity at high pHs is a desired trait for industrial applications.

The above characterization applies to typical laccases with three or more domains. We report the characterization of an enzyme that represents a new family of laccases that possesses only two domains. This laccase lacks the second domain yet exhibits significant activity. We designate the enzyme SLAC, for Small LACcase. SLAC is easily prepared following heterologous expression in *E. coli*, providing an enzyme that is simple to modify and thus a useful tool to understand activity in all laccases. Experiments show that all three copper sites are intact, and the first NMR spectrum of a laccase trinuclear cluster is presented. SLAC demonstrates resistance to detergents, high thermal stability, and is active as a dimer in both gels and in solution. The activity of SLAC against 2,6-dimethoxyphenol (DMP)

reaches a maximum at the unusually high pH of 9.4, suggesting a suitability of the enzyme for industrial processes. The pH-dependencies of DMP activity suggest that SLAC binds negatively charged substrates more tightly, and that the rate of the catalytic step is maximized at pH 8.2.

Results

Alignment

Attempts to align the entire SLAC protein (Fig. 1) against protein databases resulted in failure because of the large gap penalty of the absent middle domain. Instead, the first and last domains were each aligned, with a portion of the interdomain region (I169–H206) not included in either alignment. In addition, an insertion (R133–P147, gold-shaded region) not present in the other enzymes was also removed. Interdomain residues T207–H226 are included at the beginning of the second domain alignment to highlight the lack of homology in this region. Alignments of each domain with other laccases appear in Figure 2. Domain one of SLAC (A31–G168) has an average of 29% identity and 47% conserved homology with the laccases tested, whereas domain two (G227–H343) averages 27% identity and 45% conserved homology with the third domain of the laccases compared (EpoA not included). GenTHREADER (Jones 1999a) predicts that the protein fold of SLAC resembles the folds of both MaL and the *Coprinus cinereus* laccase (CcL) with >99% confidence. PSIPRED (Jones 1999b) forecasts the same secondary structure as measured in these laccases. It also predicts that the interdomain region consists of three strands and three large random coils (Fig. 1) without similarity to other laccase domains. SLAC has no cysteines other than the type 1 copper ligand and thus lacks the common disulfide bonds. The only other members of the laccase family that share this feature are EpoA and the six-domain protein ceruloplasmin.

One of the significant results of the alignment is that all the copper ligands are conserved, including a methionine in the predicted axial position of the type 1 site (M298). Other residues that usually form important hydrogen-bonds in the active sites are also present (D113 and D259 in SLAC correspond to D77 and D424 in TvL). It is difficult to comment on the loops that traditionally make up the substrate-binding pocket, because these motifs appear at the end of the first domain and the beginning of the third domain in other laccases. These regions have little homology within the family of proteins. The 15 amino acids (R133–P147) that were left out of alignments are located just prior to a turn normally found in the laccases at the mouth of a channel that leads to the trinuclear cluster. Because there are normally two channels to the trinuclear cluster, it is possible that the enzyme may function even if these residues block one of the openings.

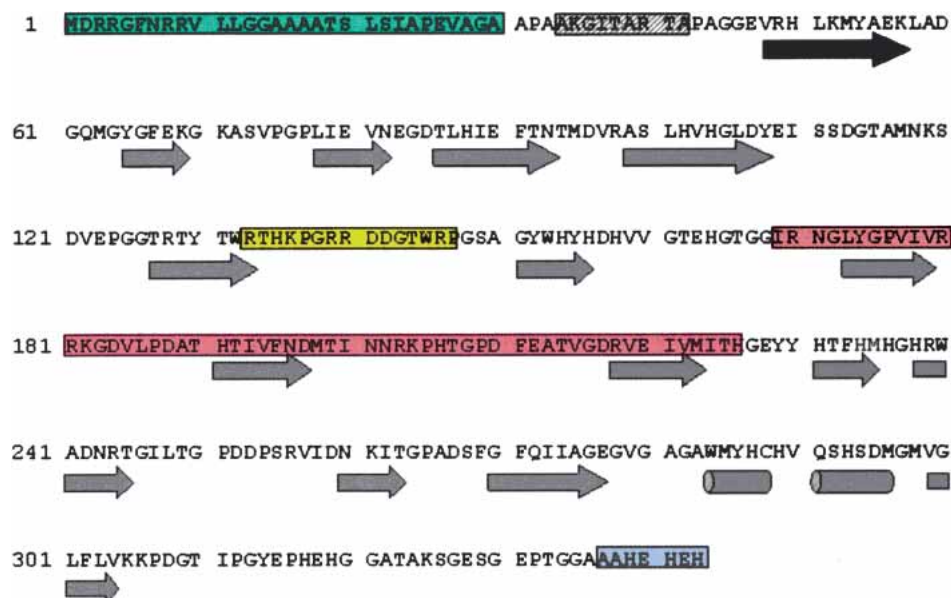


Figure 1. Analysis of the SLAC sequence. The green box contains the TAT-leader sequence (M1–A30). The hatched gray box contains the starting amino acids of the different N termini that are found in SLAC purified from *E. coli* (A34–A42). The gold box contains a loop (R133–P147) that is not normally found in laccases and occurs at a position to possibly form a flap over one of the channels leading to the trinuclear cluster. The red box is the approximate region that bridges domains one and two (I169–H226). The mauve box contains the amino acids that are cleaved from the C terminus of SLAC (A337–H343). The secondary structural predictions from PSIPRED, beginning after the TAT-leader sequence, are shown below the amino acid sequence. Arrows represent β -strands, and cylinders represent helices.

Recombinant expression and purification

To produce SLAC easily, we cloned the gene into pET20b to make pSLAC1 and transformed the final construct into BL21(DE3). Upon induction with IPTG, a band of ~32 kDa mass appears on SDS-PAGE. The presence of a *Streptomyces* TAT-leader sequence in the gene led to the expectation of SLAC in the periplasmic fraction (Berk 1996). However, all protein is found in the cytoplasm. Dialysis and anion exchange chromatography purified the protein to >97%. A dimeric band that is thermostable appears on the gels. The band representing the dimer shows activity, but the monomer is inactive (Fig. 3). Gel filtration experiments (data not shown) confirm a mass representative of a dimer of 69 ± 8 kDa. The predicted pI of the apoenzyme is 6.2, whereas the measured pI of the four-copper oxidized form was 8.2 ± 0.2 and the four-copper reduced form was 7.3 ± 0.2 . Mass spectrometry indicates a mass around 32 kDa, much lower than expected (36,875 Da). N-terminal sequencing shows that the polypeptide lacks an N-terminal stretch with varying length corresponding to the first 34–42 amino acids, and C-terminal sequencing shows that a seven-amino-acid fragment is also missing.

Streptomyces coelicolor characterization

Activity against DMP was measured for the media of the wild type and gene-disruption mutant. The wild type dem-

onstrated significant activity, whereas the gene-disruption mutant showed no activity (data not shown).

Characterization of recombinant SLAC

The first property of the enzyme that was noticed was the dramatic blue color of the holoenzyme that is generated by convection of copper-incubated solution with the atmosphere. The type 1 site appears typical in UV-visible ($\lambda_{\text{max}} = 590$ nm, $\epsilon = 4400$ M⁻¹ cm⁻¹) spectra and demonstrates an axial EPR ($g_{\parallel} = 2.23$, $A_{\parallel} = 85 \times 10^{-4}$ cm⁻¹) spectrum (Fig. 4). The so-called type 2 copper is EPR-detectable and also axial, but its overlap with the type 1 signal prevents accurate determination of the g -values or the hyperfine splitting. The type 3 copper site dinuclear transition is seen in the UV-visible spectra at 330 nm. BCA quantitation finds 4.0 ± 0.4 copper ions per SLAC monomer, which is confirmed by atomic absorption. The quantification and spectroscopy show that the four copper ions common to laccases and required for activity are present. We have also measured the paramagnetic NMR of the protein. We discern several paramagnetically shifted peaks due to the copper sites, including several with anti-Curie behavior (Fig. 5).

Activity assays

We observed that SLAC has activity against a wide range of substrates, including *t*-butyl catechol, 3-(3,4-dihydroxyph-

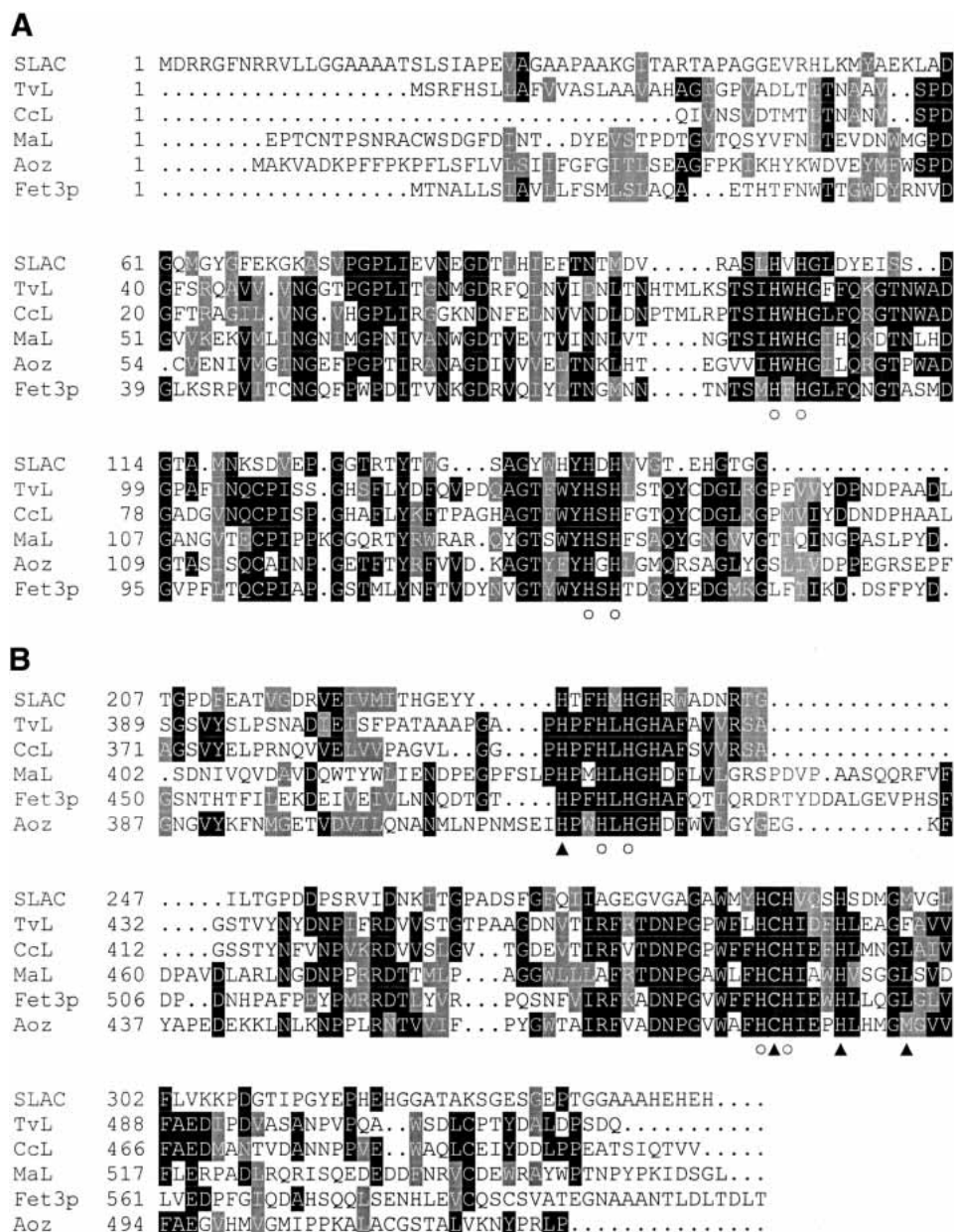


Figure 2. (A) Multiple sequence alignment of SLAC domain one with the first domain of other laccases. The proteins and the accession numbers are SLAC (CAB45586), *Trametes versicolor* laccase (TvL, A35883), *Coprinus cinereus* laccase (CcL, 1A65A), *Melanocarpus albomyces* laccase (MaL, 1GW0A), *Saccharomyces cerevisiae* Fet3p (Fet3p, CAA89768), and cucumber ascorbate oxidase (Aoz, KSKVAO). Ligands to the type 1 copper site are indicated by filled triangles at the bottom of their columns, and ligands to the trinuclear cluster are indicated by open circles. Black-shaded regions highlight identical amino acids, and gray-shaded regions highlight regions of conserved homology. (B) Multiple sequence alignment of the second domain of SLAC with the third domains of three-domain laccases.

nyl)-L-alanine (DOPA), syringaldazine (SGZ), DMP, and $\text{Fe}(\text{CN})_6^{4-}$. The reaction velocities are linear with respect to enzyme concentration. Interactions with DMP and $\text{Fe}(\text{CN})_6^{4-}$ were selected for further characterization; DMP is a member of the phenolic class of substrates that is particularly stable against autooxidation at high pH (because of

its high pK_a value), and $\text{Fe}(\text{CN})_6^{4-}$ represents nonphenolic substrates. This distinction is important, as the reduction potentials of phenols have a marked pH-dependence. Aqueous iron(II), a substrate of the iron oxidoreductase-type laccases, does not react with SLAC. In contrast, the negatively charged ferrocyanide complex is rapidly oxidized. The K_m

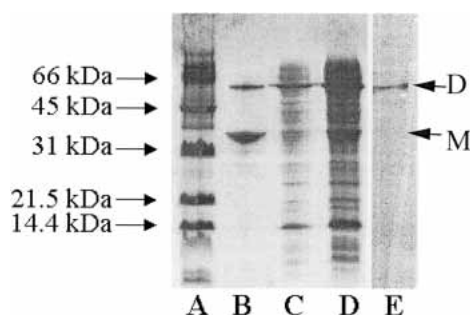


Figure 3. SDS-PAGE gel of SLAC expression, purification, and activity. Lanes A–D are stained with Coomassie blue. (A) Molecular weight standard. (B) Purified SLAC—both monomer and dimer bands are observed. (C) Diluted copper-incubated cytoplasmic fraction of *E. coli* that has been transformed with pSLAC1, grown, and induced. (D) Cytoplasmic fraction from C directly after cell lysis and before dilution and copper incubation shows both monomeric and dimeric bands. (E) Purified SLAC, identical to lane B, stained by activity against DOPA, showing that activity arises from the dimer. The labels D and M on the right side of the gel designate the positions of the dimer and the monomer bands, respectively.

for ferrocyanide is constant ($\sim 400 \mu\text{M}$) over the short pH range ($4.5 < \text{pH} < 5.5$) studied. The pH range is limited by protein instability in the acidic regime and substrate auto-oxidation at high concentrations of ferrocyanide at more alkaline pHs. The k_{cat} of $\text{Fe}(\text{CN})_6^{4-}$ decreases with an increase in pH, similar to that observed in all other laccases (Fig. 6).

The K_m for DMP is pH-dependent, decreasing as the pH increases (Fig. 7). The binding behavior of DMP is consistent with pK_a values of 8.3 ± 0.1 for the bound form and 9.5 ± 0.3 for the free form. Thus, the phenolate is bound more tightly than the phenol. The pK_a value of free DMP, 9.6, is in good agreement with this analysis (Xu 1996). The k_{cat} of DMP reaches a maximum at approximately pH 8.2, with the standard bell-shaped dependence seen for other laccases. The derived pK_a values are 7.3 ± 0.1 and 9.2 ± 0.1 for the enzyme–DMP complex. The maximum of k_{cat}/K_m for DMP occurs at pH 9.4, which is the most alkaline pH optimum measured for laccases.

Discussion

Multiple sequence alignment clearly shows that the ligands for the type 1 site and trinuclear cluster are present, and located in the same relative positions as found in other laccases. Secondary structure prediction and comparisons find significant alignment of the two domains of SLAC with cupredoxin folds. It has been hypothesized that the cupredoxin domain has been often recycled for various purposes, from copper nitrite reductase to the human coagulation factor VIII (Murphy et al. 1997). One of the most important similarities between these enzymes is the position and identity of the metal ligands. The alignment shows that all the copper ligands are conserved in their proper locations. The

spectroscopy and copper quantitation validate the presence and structure of the three expected copper sites.

One unusual feature is the presence of 24 histidines in the full sequence. If 10 residues are the expected copper ligands, 14 are left. These 14 residues represent 4.6% of SLAC's amino acids, about double the average histidine composition (2.26%) of proteins (Gasteiger et al. 2001). CueO from *E. coli* has a high percentage of methionine residues and has been suggested to play a role in resistance to copper toxicity (Roberts et al. 2002). It is possible that a secondary function of SLAC is to bind a surplus of intracellular copper and carry it when exported by the TAT secretory apparatus. Extensive *in vivo* work is currently being done to address this question.

The first 30 amino acids encoded by the SLAC gene form a twin-arginine-targeted (TAT) leader sequence that signals the protein for export via the folded-protein pathway. Many laccase proteins are transported in a similar manner, including the *E. coli* proteins CueO and PcoA (Lee et al. 2002). When expressed recombinantly in *E. coli*, SLAC is found in the cytoplasm and the N-terminal residue is one of several occurring downstream from the TAT-leader sequence. These results suggest that SLAC is degraded in the cytoplasm of *E. coli*. However, in cultures of *S. coelicolor*, laccase activity is detected in the media. Laccase activity is not present in the strain with the SLAC gene disrupted, indicating that SLAC is exported when expressed homologously and that SLAC is the primary extracellular laccase.

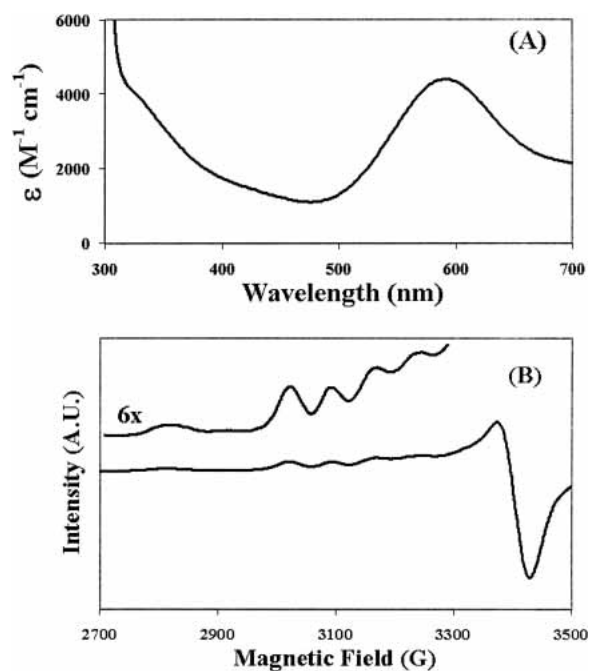


Figure 4. (A) UV-visible absorption spectrum of SLAC. The extinction coefficient, ϵ , is plotted on the ordinate axis. (B) Plot of the derivative of the EPR spectrum of SLAC, with an expansion of the $g_{||}$ region inset.

The lack of the second domain that is normally found in laccases raises several issues. One such issue is the question of how the two domains are connected. Normally, a short loop or helix of nine amino acids connects domains one and two, but domains two and three are usually linked by a long coil of 40 or more amino acids. Laccases have very low sequence homology in this coil. It is thus not surprising that the two domains of SLAC are connected by ~60 amino acids of unknown structure. This section of SLAC also would include all of the substrate-binding features from a full-size laccase. The 60-amino-acid segment is predicted to be almost neutral at pH 7.0, and negative charges seem randomly distributed. There are two small clusters of positive residues (following R180 and R203), perhaps near a substrate-binding site.

Another issue regarding the absent domain is "Can the enzyme function without this highly conserved element?" It clearly does but only in the dimeric form, as predicted by Murphy et al. (1997). The crystal structure of the ascorbate oxidase dimer from cucumber shows that 32 of the 39 residues that form the dimer interface are in domains one and three (Messerschmidt et al. 1992). Domain two frequently

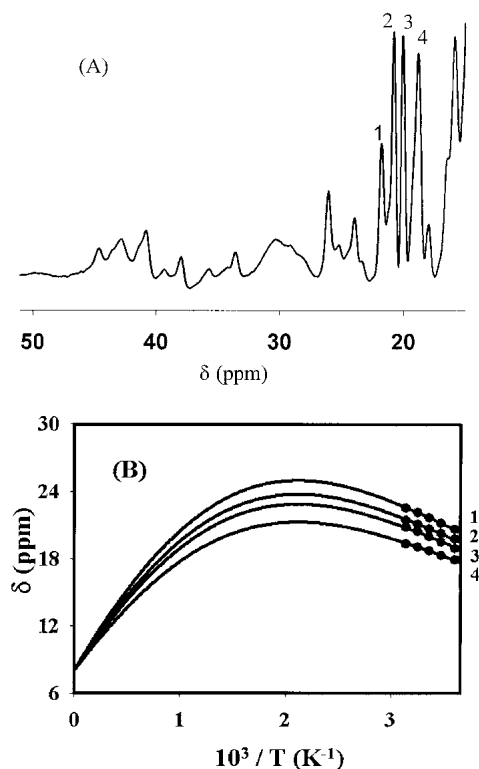


Figure 5. (A) Downfield region of the super-WEFT NMR spectrum of SLAC, showing paramagnetically shifted peaks. (B) The temperature dependencies of peaks 1–4 demonstrate anti-Curie behavior. The filled circles are data points, and the lines represent the best fits to the data using nonlinear regressions of equation 1.

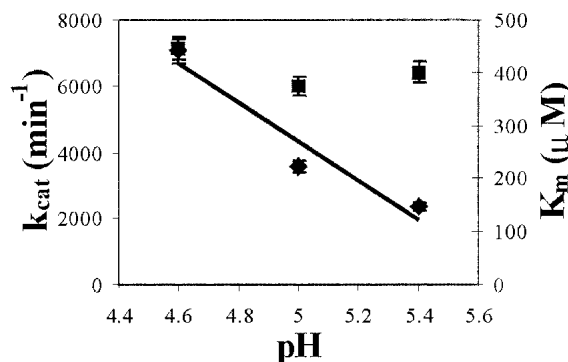


Figure 6. Plots of the Michaelis-Menten constant, K_m (squares, right axis), and the k_{cat} (diamonds, left axis) for the reaction of SLAC with ferrocyanide vs. pH. The line represents a linear regression of the data points for k_{cat} .

contributes several residues to the substrate pocket, but other residues that form the binding cleft near the type 1 copper are provided by loops and turns between domain two and domains one and three. These comparisons show that although the majority of the surface for dimer formation is present, the machinery for binding the reducing substrate must have been reengineered. An alternative explanation could be that the copper ions of the trinuclear cluster bridge the first domain of one monomer and the second domain of a second monomer, so that the two monomers form a head-to-tail arrangement. Finally, it is possible that the monomeric, folded form has activity, but that this species is not observed in solution or on SDS-PAGE gels.

The interactions for dimer formation may still be present, but another two-domain copper enzyme, the nitrite reductase from *Alcaligenes faecalis*, naturally forms a homotrimer. EpoA of *S. griseus*, the only other characterized example of a laccase lacking the second domain, is 69% identical to SLAC. Despite this near identity, EpoA is also active as a trimer (Endo et al. 2002). It is possible that agglomeration occurs with the high percentage of histidines and copper, although such behavior is not witnessed in SLAC.

The activity of SLAC against DMP is striking in that it reaches an optimum at an alkaline pH. Quite often, phenol-oxidase activity is measured at acidic pHs (4.0–5.0), and a few enzymes peak at pH 7.0–8.0 (Table 1). Mutational analyses on several laccases suggest that an acidic residue (analogous to D295 in SLAC) raises the optimum of *Acremonium* ascorbate oxidase by one pH unit, but the reported differences in activity are very small, that is, the top of the bell-shaped curve of activity versus pH is almost flat (Sugino et al. 2002). The increase of activity with pH has been attributed to a decrease in substrate reduction potential that is favorable for electron transfer to the type 1 copper site (Xu 1997). The other half of the bell curve, representing a decrease of activity with pH, has been attributed to hydroxide inhibition at the trinuclear cluster.

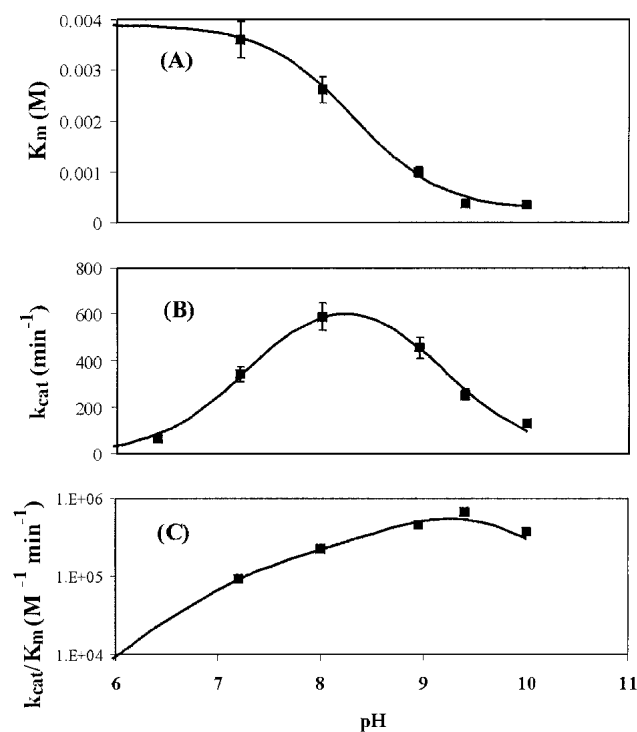


Figure 7. Plots of the Michaelis-Menten constant (A) K_m , (B) k_{cat} , and (C) the ratio k_{cat}/K_m for the reaction of SLAC with DMP vs. pH. The lines represent the best fits to the data using nonlinear regressions of equations 2, 3, and the quotient of equation 3 divided by equation 2.

The dependencies of k_{cat} and K_m on pH in this work form a different understanding for the reactivity of SLAC. The decrease of K_m with increasing pH reflects the binding of the anionic form of DMP, with a commensurate correlation to the substrate's pK_a value. That ferrocyanide and not aqueous iron(II) is bound by and reacts with SLAC confirms that the enzyme prefers to bind negative substrates over uncharged or positive ones. It is possible that the two positive clusters of residues in the interdomain linker are responsible for this behavior. Also, it is usually the situation that the variation in k_{cat}/K_m mirrors the trend in k_{cat} near the pH of optimum activity. Here, in contrast, the binding of substrate to SLAC dominates k_{cat}/K_m until the pK_a value of the free substrate is reached and the pH dependency of K_m levels off.

The catalysis of phenol oxidation mirrors the results of three-domain laccases. From pH 6.4 to pH 10.0, the catalytic rate constant takes the shape of a bell curve (Xu 1997). Optimum activity is consistent with a deprotonation at pK_a 7.3 and a protonation at pK_a 9.2. It is important to note that these pK_a values correspond to the bound enzyme–substrate complex (the species responsible for catalytic activity), and not to the parameters of the free substrate that are calculated from fitting K_m . Also, because neither of these pK_a values corresponds to the pK_a value of the enzyme–substrate com-

plex (8.3) that is derived from the substrate-binding behavior, they are likely to involve changes in a separate part of the protein: the trinuclear cluster. This hypothesis is in concordance with previous work that ascribes a reduction in activity to hydroxide inhibition at the trinuclear cluster.

Rough correlations have also been made between the pI of laccases and their optimal pH for SGZ oxidation (Xu et al. 1996). From the measured pI of 8.2, the optimal pH should be above 8.0, second only to *R. vernicifera* laccase. The fact that a high pH optimum is seen in SLAC for DMP, a similar substrate to SGZ, is further support for this correlation. Similar correlations between the k_{cat} and the reduction potentials of laccases have been made. The values of k_{cat} for DMP oxidation measured in this study suggest that SLAC is a “low-potential” laccase, with the reduction potential of the type 1 site near 0.5 V versus normal hydrogen electrode.

Paramagnetic NMR of copper proteins, primarily blue copper proteins, has been studied for more than two decades. A mononuclear copper(II) center typically exhibits Curie behavior, or an increase in chemical shift with a decrease in temperature. Recently, this lab began to characterize tyrosinase, a protein with a type 3 dinuclear copper center, which shows anti-Curie behavior as a result of antiferromagnetic coupling between the two copper ions (Bubbico et al. 1999; Tepper et al. 2002). Herein, we have reported the paramagnetic NMR spectrum of a laccase, which has recently been also reported by Battistuzzi et al. (2003). The resonances in the NMR spectrum of that work are assigned to the mononuclear type 1 site; in contrast, the anti-Curie behavior of several of the peaks in SLAC shows that, for the first time, the trinuclear cluster of a laccase has been observed with NMR. The characterization of these spectra is the subject of another manuscript.

A novel laccase enzyme has been cloned and characterized, and is denoted “small laccase” (SLAC) because it lacks a domain and is thus much smaller than other laccases.

Table 1. pH optima of DMP and SGZ oxidase activity for several laccases

Organism	Enzyme	pH	Substrate	Reference
<i>E. coli</i>	CueO	5.5, 8	DMP	Roberts et al. 2003
<i>T. pubescens</i>	Laccase	3–4.5	DMP, SGZ	Galhaup et al. 2002
<i>B. subtilis</i>	CotA	7.0	SGZ	Martins et al. 2002
<i>P. cinnabarinus</i>	Laccase	4	SGZ	Record et al. 2002
<i>P. pinsitus</i>	Laccase	5	SGZ	Xu et al. 1996
<i>R. solani</i>	Laccase	7	SGZ	Xu et al. 1996
<i>M. thermophila</i>	Laccase	6	SGZ	Xu et al. 1996
<i>S. thermophilum</i>	Laccase	7	SGZ	Xu et al. 1996
<i>R. vernicifera</i>	Laccase	9	SGZ	Xu et al. 1996
<i>M. verrucaria</i>	Bilirubin oxidase	8	SGZ	Xu et al. 1996

Despite the lack of this domain, the enzyme maintains the normal complement of four copper ions and is active against the common range of substrates, preferring to bind those that are negatively charged. A group of ~60 amino acids takes the place of the substrate-binding loops that are found in three-domain laccases. The protein is functional in solution and denaturing gels as a dimer, and converts phenols effectively at unprecedentedly high pHs. It also appears to be the primary extracellular laccase in its host organism, *S. coelicolor*. These many features, coupled with the ease of preparation of SLAC, suggest that the enzyme will be a useful implement for understanding the properties of laccases.

Materials and methods

Recombinant expression and purification of SLAC

A 1.4-kb fragment containing the gene encoding SLAC was generated by PCR with genomic DNA of *S. coelicolor* M145 as template and the following primers: CGCGAATTCATATGGACAG GCGAGGCTTTAACCGACG (MCOF) and CGCAAGCTTCTC CGGTTCCGCGGCGACG (MCOR). The forward primer, MCOF, introduced an NdeI site (underlined) on the start codon (italics) and an EcoRI site (bold), whereas the reverse primer, MCOR, introduced a HindIII (underlined) site 420 bp after the stop codon. The NdeI/HindIII-digested PCR fragment was cloned into pET 20b (Novagen), generating pSLAC1, which was transformed into *E. coli* BL21(DE3). Transformants were selected on LB media containing 100 µg/mL ampicillin. DNA sequencing (Baseclear) confirmed the sequence of the cloned gene.

For medium-scale production of SLAC, 3 L of 2xYT media was inoculated with 30 mL of an overnight culture. The cells were grown at 30°C until reaching OD₆₀₀ ~ 1.5, when the temperature was reduced to 25°C and the cells were induced with 0.4 mM isopropyl-β-D-thiogalactopyranoside (Eurogentec). After 20 h of growth, the cells were collected by centrifugation for 10 min at 5000g. The pellet was resuspended in 150 mL of 10 mM NaP_i (pH 7.3), and the resulting solution was sonicated to lyse the cells. The soluble fraction was obtained by centrifuging for 20 min at 27,000g. The soluble fraction was incubated for several hours with 1 mM Cu(SO₄), at which time 150 µg of DNAase I (Roche) and 10 µg of RNAase (Eurogentec) were added.

The solution was then dialyzed four times against 5 L of the same buffer that was used to suspend the cell pellets. Then 1 mM EDTA was added to the second dialysis to remove excess copper from the protein. The solution was then applied to a diethylaminoethyl (DEAE) column (100 mm long by 50 mm diameter; Amersham Pharmacia) that had been equilibrated with the same phosphate buffer. A step gradient elutes the pure SLAC protein at 100 mM NaCl. The blue fractions were pooled and concentrated, and the purity was determined by SDS-PAGE and UV-visible absorbance measurements ($A_{280\text{ nm}}/A_{590\text{ nm}} < 11$ indicates >97% purity). Protein concentration was initially assessed using both the Bradford technique and the absorbance at 280 nm of the unfolded protein in 6 M guanidinium hydrochloride (Gill and von Hippel 1989); these concentrations were then used to determine the extinction coefficients for the 280 nm, 330 nm, and 590 nm peaks of the folded protein. The folded-protein extinction coefficients were used in all other measurements. When testing for export of the

protein, the periplasmic fraction of the *E. coli* was isolated by osmotic shock.

Other methods

The amount of copper bound to the protein was typically determined through the trichloroacetic acid/bicinchoninic acid (BCA) method of Brenner and Harris (1995) and confirmed by atomic absorption (Department of Toxicology, Leiden University Medical Center). The isoelectric point of the protein was determined using IEF gels on a Phast System (Amersham Biosciences) with broad pI standards. Reduced protein was prepared by incubation with ascorbic acid or sodium dithionite. For gel filtration, an aliquot of protein was injected onto a Superose 12 column (Amersham Biosciences) that had been equilibrated with 10 mM NaP_i (pH 7.3), and the elution volume was compared against a series of standards (Sigma): blue dextran (2000 kDa), β-amylase (200 kDa), alcohol dehydrogenase (150 kDa), bovine serum albumin (66 kDa dimer), carbonic anhydrase (29 kDa), cytochrome *c* (12 kDa), and tryptophan (0.17 kDa).

Electron paramagnetic resonance (EPR)

EPR measurements were made at 40 K on a Bruker Eleksys 680 (Bruker Biospin GmbH Rheinstetten) spectrometer operating at 9.73 GHz (X-band). The protein sample contained 1.5 mM SLAC in 30% glycerol, 70% 100 mM NaP_i buffer (pH 7.2). The instrument parameters were microwave power, 19.75 µW; modulation frequency, 100 kHz; modulation amplitude, 0.5 mT; time constant, 2.56 msec; sweep rate, 19.1 mT/sec.

Nuclear magnetic resonance

NMR samples of fresh protein (1 mM) were prepared in 100 mM NaP_i buffer (pH 7.3) in H₂O/D₂O (90/10 v/v). ¹H NMR spectra were recorded on a Bruker DMX-600 NMR spectrometer using a super-WEFT pulse sequence (interpulse delay, 100 msec; repetition rate, 6 sec⁻¹; spectral width, 150 ppm). In all, 32,000 free induction decays were acquired and Fourier-transformed using a Gaussian window (LB = -10 Hz, GB = 0.02) and baseline-corrected using Bruker-provided software. The temperature-dependence of the paramagnetic shift of the peaks was fit to the following equation, which assumes that two copper ions are antiferromagnetically coupled with an exchange coupling constant *J*, yielding a diamagnetic ground state with an excited paramagnetic (*S* = 1) state -2*J* in energy above the ground state (Bubacco et al. 1999):

$$\delta = \frac{2Ag\beta H}{\hbar k_B T} \frac{\exp(2J/k_B T)}{1 + 3 \exp(2J/k_B T)} \quad (1)$$

In this equation, β is the Bohr magneton, ħ is Planck's constant divided by 2π, *k_B* is Boltzmann's constant, *T* is the absolute temperature, *H* is the externally applied magnetic field, and *A* is the isotropic dipolar interaction energy between the magnetic moment of the electrons and the nuclear magnetic moment.

Activity assays

Activity assays were typically carried out in 100 mM acetate (pH 4.0–6.0), NaP_i (pH 6.0–8.0), Tris (pH 7.0–9.0), or carbonate (pH

9.0–11.0) buffers. DMP turnover was monitored at 468 nm ($\epsilon = 14,800 \text{ M}^{-1} \text{ cm}^{-1}$ for the dimeric product; Solano et al. 2001), $\text{Fe}(\text{CN})_6^{4-}$ at 405 nm ($\epsilon = 900 \text{ M}^{-1} \text{ cm}^{-1}$), and $\text{Fe}_{\text{aq}}^{2+}$ at 315 nm ($\epsilon = 2200 \text{ M}^{-1} \text{ cm}^{-1}$). All UV-visible absorption measurements were obtained using a Shimadzu UV-2101PC spectrophotometer. The method of initial rates was used to determine the substrate-dependent reaction velocities, which were then fit with the Michaelis-Menten equation. The pH-dependencies of the catalytic rate constant (k_{cat}) and Michaelis-Menten constant (K_{m}) were fit using equations 2 and 3 (Segel 1975):

$$(K_{\text{M}})_{\text{H}} = K_{\text{D}} K_{\text{ES}} \frac{(1 + [\text{H}^+] / K_{\text{S}})}{K_{\text{ES}} + [\text{H}^+]} \quad (2)$$

$$(k_{\text{cat}})_{\text{H}} = (k_{\text{cat}})_{\text{max}} \frac{1}{(1 + ([\text{H}^+] / K_{\text{ES1}}) + (K_{\text{ES2}} / [\text{H}^+]))} \quad (3)$$

In equation 2, K_{D} is the dissociation constant of the substrate in the deprotonated form, K_{ES} is an acid dissociation constant for the enzyme–substrate complex, and K_{S} is the acid dissociation constant for the free substrate. In equation 3, K_{ES1} and K_{ES2} are acid dissociation constants for the enzyme–substrate complex (these constants are unrelated to K_{ES} in equation 2).

Streptomyces strains and growth

M145 (SCP1-, SCP2-) was used for all *Streptomyces* experiments, except where the disruption mutant was used (vide infra). Spores, mycelia, and protoplasts were all manipulated by established protocols (Kieser et al. 2000). Disruption of the SLAC gene in M145 by the insertion of an apramycin-resistance cassette was accomplished by PCR-targeted gene replacement (Gust et al. 2003). The cosmid 4C6 was used for recombination, and the PCR primers used were TCTGCGCACATTCCGTATCGGCGTGGGGAGTTC GGCATGATTCCGGGGATCCGTCGACC (MCOKOF) and CC GGCGGCCCGGTGGCGCGGCCGCGGCCCGGCCGCTCAT GTAGGCTGGAGCTGCTTC (MCOKOR). Disruption was confirmed by Southern blot.

Homologous expression and localization

Cultures of M145 and the ΔSLAC strain were seeded with freshly harvested spores and grown for 5 d at 30°C in flasks containing metal springs and liquid R5 media without antibiotics. Mycelia were harvested by centrifugation (3000g for 10 min). DMP oxidase activity of the media was determined under the following conditions: 50 μL of growth medium was added to 950 μL of a 2.5 mM solution of DMP with 100 mM Tris buffer at pH 8.0. Absorption at 468 nm was monitored with time.

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References

- Battistuzzi, G., Di Rocco, G., Leonardi, A., and Sola, M. 2003. ^1H NMR of native and azide-inhibited laccase from *Rhus vernicifera*. *J. Inorg. Biochem.* **96**: 503–506.
- Berks, B.C. 1996. A common export pathway for proteins binding complex redox cofactors? *Mol. Microbiol.* **22**: 393–404.
- Brenner, A.J. and Harris, E.D. 1995. A quantitative test for copper using bicinchoninic acid. *Anal. Biochem.* **226**: 80–84.
- Bubacco, L., Salgado, J., Tepper, A.W.J.W., Vijgenboom, E., and Canters, G.W. 1999. ^1H NMR spectroscopy of the binuclear $\text{Cu}(\text{II})$ active site of *Streptomyces antibioticus* tyrosinase. *FEBS Lett.* **442**: 215–220.
- Claus, H. 2003. Laccases and their occurrence in prokaryotes. *Arch. Microbiol.* **179**: 145–150.
- Edens, W.A., Goins, T.Q., Dooley, D., and Henson, J.M. 1999. Purification and characterization of a secreted laccase of *Gaeumannomyces graminis* var. *tritici*. *Appl. Environ. Microbiol.* **65**: 3071–3074.
- Endo, K., Hosono, K., Beppu, T., and Ueda, K. 2002. A novel extracytoplasmic phenol oxidase of *Streptomyces*: Its possible involvement in the onset of morphogenesis. *Microbiology* **148**: 1767–1776.
- Galhaup, C., Goller, S., Peterbauer, C.K., Strauss, J., and Haltrich, D. 2002. Characterization of the major laccase isoenzyme from *Trametes pubescens* and regulation of its synthesis by metal ions. *Microbiology* **148**: 2159–2169.
- Gasteiger, E., Jung, E., and Bairoch, A. 2001. SWISS-PROT: Connecting bio-molecular knowledge via a protein database. *Curr. Issues Mol. Biol.* **3**: 47–55.
- Gill, S.C. and von Hippel, P.H. 1989. Calculation of protein extinction coefficients from amino acid sequence data. *Anal. Biochem.* **182**: 319–326.
- Gust, B., Challis, G.L., Fowler, K., Kieser, T., and Chater, K.F. 2003. PCR-targeted *Streptomyces* gene replacement identifies a protein domain needed for biosynthesis of the sesquiterpene soil odor geosmin. *Proc. Natl. Acad. Sci.* **100**: 1541–1546.
- Hakulinen, N., Kiiskinen, L., Kruus, K., Saloheimo, M., Paananen, A., Koivula, A., and Rouvinen, J. 2002. Crystal structure of a laccase from *Melanocarpus albomyces* with an intact trinuclear site. *Nat. Struct. Biol.* **9**: 601–605.
- Jones, D.T. 1999a. GenTHREADER: An efficient and reliable protein fold recognition method for genomic sequences. *J. Mol. Biol.* **287**: 797–815.
- . 1999b. Protein secondary structure prediction based on position-specific scoring matrices. *J. Mol. Biol.* **292**: 195–202.
- Kieser, T., Bibb, M.J., Buttner, M.J., Chater, K.F., and Hopwood, D.A. 2000. *Practical Streptomyces genetics*. The John Innes Foundation, Norwich UK.
- Lee, S.M., Grass, G., Rensing, C., Barrett, S.R., Yates, C.J., Stoyanov, J.V., and Brown, N.L. 2002. The Pco proteins are involved in periplasmic copper handling in *Escherichia coli*. *Biochem. Biophys. Res. Commun.* **295**: 616–620.
- Martins, L.O., Soares, C.M., Pereira, M.M., Teixeira, M., Costa, T., Jones, G.H., and Henriques, A.O. 2002. Molecular and biochemical characterization of a highly stable bacterial laccase that occurs as a structural component of the *Bacillus subtilis* endospore coat. *J. Biol. Chem.* **277**: 18849–18859.
- Mayer, A.M. and Staples, R.C. 2002. Laccase: New functions for an old enzyme. *Phytochemistry* **60**: 551–565.
- Messerschmidt, A., ed. 1997. *Multi-copper oxidases*. World Scientific, Singapore.
- Messerschmidt, A. and Huber, R. 1990. The blue oxidases, ascorbate oxidase, laccase and ceruloplasmin. Modelling and structural relationships. *Eur. J. Biochem.* **187**: 341–352.
- Messerschmidt, A., Ladenstein, R., Huber, R., Bolognesi, M., Avigliano, L., Petruzzelli, R., Rossi, A., and Finazzi-Agro, A. 1992. Refined crystal structure of ascorbate oxidase at 1.9 Å resolution. *J. Mol. Biol.* **224**: 179–205.
- Murphy, M.E.P., Lindley, P.F., and Adman, E.T. 1997. Structural comparison of cupredoxin domains: Domain recycling to construct proteins with novel functions. *Protein Sci.* **6**: 761–770.
- Piontek, K., Antorini, M., and Choinowski, T. 2002. Crystal structure of a laccase from the fungus *Trametes versicolor* at 1.90-Å resolution containing a full complement of coppers. *J. Biol. Chem.* **277**: 37663–37669.
- Record, E., Punt, P.J., Chamkha, M., Labat, M., van den Hondel, C.A.M.J.J., and Asther, M. 2002. Expression of the *Pycnoporus cinnabarinus* laccase gene in *Aspergillus niger* and characterization of the recombinant enzyme. *Eur. J. Biochem.* **269**: 602–609.
- Rensing, C. and Grass, G. 2003. *Escherichia coli* mechanisms of copper homeostasis in a changing environment. *FEMS Microbiol. Rev.* **27**: 197–213.
- Roberts, S.A., Weichsel, A., Grass, G., Thakali, K., and Hazzard, J.T. 2002. Crystal structure and electron transfer kinetics of CueO, a multicopper oxidase required for copper homeostasis in *Escherichia coli*. *Proc. Natl. Acad. Sci.* **99**: 2766–2771.
- Roberts, S.A., Wildner, G.F., Grass, G., Weichsel, A., Ambrus, A., Rensing, C.,

- and Montfort, W.R. 2003. A labile regulatory copper ion lies near the T1 copper site in the multicopper oxidase CueO. *J. Biol. Chem.* **278**: 31958–31963.
- Segel, I.H. 1975. *Enzyme kinetics*. John Wiley & Sons, New York.
- Solano, F., Lucas-Elio, P., López-Serrano, D., Fernández, E., and Sanchez-Amat, A. 2001. Dimethoxyphenol oxidase activity of different microbial blue multicopper proteins. *FEMS Microbiol. Lett.* **204**: 175–181.
- Solomon, E.I., Sundaram, U.M., and Machonkin, T.E. 1996. Multicopper oxidases and oxygenases. *Chem. Rev.* **96**: 2563–2605.
- Sugino, M., Kajita, S., Banno, K., Shirai, T., Yamane, T., Kato, M., Kobayashi, T., and Tsukagoshi, N. 2002. Upward shift of the pH optimum of *Acremonium* ascorbate oxidase. *Biochim. Biophys. Acta* **1596**: 36–46.
- Tepper, A.W.J.W., Bubacco, L., and Canters, G.W. 2002. Structural basis and mechanism of the inhibition of the type-3 copper protein tyrosinase from *Streptomyces antibioticus* by halide ions. *J. Biol. Chem.* **277**: 30436–30444.
- Xu, F. 1996. Oxidation of phenols, anilines, and benzenethiols by fungal laccases: Correlation between activity and redox potentials as well as halide inhibition. *Biochemistry* **35**: 7608–7614.
- . 1997. Effects of redox potential and hydroxide inhibition on the pH activity profile of fungal laccases. *J. Biol. Chem.* **272**: 924–928.
- Xu, F., Shin, W., Brown, S.H., Wahleithner, J.A., Sundaram, U.M., and Solomon, E.I. 1996. A study of a series of recombinant fungal laccases and bilirubin oxidase that exhibit significant differences in redox potential, substrate specificity, and stability. *Biochim. Biophys. Acta* **1292**: 303–311.
- Yoshida, H. 1883. Chemistry of lacquer (*Urushi*) part 1. *J. Chem. Soc.* **43**: 472–486.