Copper Proteins and Copper Enzymes

Volume III

Editor

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PREFACE

These volumes of *Copper Proteins and Copper Enzymes* are intended to describe the contemporary spectroscopy and other biophysical chemistry now being applied to copper proteins in order to determine the structures of their active sites. Several chapters of the treatise describe the functional understanding which is emerging from the new work. The authors are all major contributors to research progress on copper proteins and the volumes will be found to be definitive and authoritative.

The subject, copper proteins and copper enzymes, is a very lively one and is best considered in the broadest biological and chemical contexts as it continues to develop. Copper itself, born in the dust of the cosmos and comprising perhaps 0.007% of the earth's crust, occurs in the biosphere as about two dozen families of copper proteins which serve to transport O_2, to activate it toward reaction with organic molecules, and to transfer electrons between donors and acceptors. The families of copper proteins include the azurins, plastocyanins, metallothioneins, superoxide dismutases, ceruloplasmins, laccases, ascorbate oxidases, cytochrome *c* oxidases, monoamine oxidases, diamine oxidases, galactose oxidase, hexose oxidase, urate oxidases, polyphenol oxidases, phenol *o*-hydroxylases, *p*-coumarate 3-monooxygenase, dopamine β-monooxygenase, lysine protocollagen oxygenase, quercetin 2,3-dioxygenase, and the arthropodan and molluscan hemocyanins. The number of types of copper-binding domains in these proteins appears to be very limited, perhaps three or four (blue type-1 mononuclear copper sites; type-2 mononuclear copper sites; diamagnetic binuclear type-3 copper sites), and it is probable that there are very few evolutionary prototypes from which the existing families sprung.

The structures of the copper-binding domains are being rapidly worked out, as these volumes will demonstrate. However, the overall three-dimensional protein structures are in general not known, although progress is being made. The three-dimensional structures will represent major opportunities to understand the chemical biology of these proteins because their functional properties almost certainly depend upon the presence of structural domains other than the copper-binding ones; e.g., the affinity of the copper sites for oxygen may be strongly affected by the kind and disposition of amino-acid residues. In the case of hemoglobin, almost 90 abnormal hemoglobins are known to arise from amino-acid substitutions, which show altered O_2 affinities. Since the K_m (O_2) of copper enzymes is an adaptive property, it is likely that the structures of the active sites will vary in space even though the ligands remain the same for each type.

Another interesting problem against which the information in these volumes should be weighed lies in the fact that each of the functions served by copper proteins is also served by families of iron-, heme-, and flavin-containing proteins. Why then was copper selected when other prosthetic groups were available?

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He was born in Louvain in 1920 and educated there. He received his doctorate in physical chemistry (with Professor J. C. Jungers) from the Katholieke Universiteit in 1942. He was trained in protein chemistry as a Research Assistant and Senior Research Assistant of the National Fund for Scientific Research (Belgium) at the Laboratorium voor Biochemie in Louvain under Professor P. Putzeys. As a Graduate Fellow of the Belgian American Educational Foundation he was a Research Fellow in Physical Chemistry at the Department of Physical Chemistry, Harvard Medical School, Boston, Mass. (Professor E. J. Cohn, Professor J. T. Edsall, Professor J. L. Oncley).

He was President of the Vlaamse Chemische Vereniging and of the Belgische Vereniging voor Biochemie — Société Belge de Biochimie. He is a fellow of the American Association for the Advancement of Science, a member of the Royal Society of Sciences of Uppsala, Sweden, the New York Academy of Science, the American Chemical Society, the Biochemical Society (London), the Société de Chimie biologique (Paris), the Society of the Sigma Xi. He is member of the Advisory Board of the *European Journal of Biochemistry* and of *Inorganica Chemica Acta,* Bioinorganic Chemistry, Articles and Letters.

His major research interests, which included milk and barley proteins, are focused now on copper proteins, mainly on the structure, function, and biosynthesis of hemocyanins.

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COPPER PROTEINS AND COPPER ENZYMES

René Lontie

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Chapter 1

LACCASE

Bengt Reinhammar

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I. INTRODUCTION

Laccase is a blue copper-containing oxidase which is widely distributed in higher plants and fungi. It belongs to a small group of blue oxidases which can utilize the full oxidizing capacity of dioxygen and reduce it to two molecules of water. The other enzymes are the blue proteins ceruloplasmin and ascorbate oxidase which have many properties in common with laccase. They will, however, not be considered here since they are treated in separate chapters in this volume.

Laccase is one of the most complicated copper-containing oxidases as it contains three types of quite unique copper ions. Nevertheless, it has been extensively studied by a number of different techniques. Therefore, it is one of the best understood oxidases, at least in terms of its catalytic mechanism.

It is the intention of this chapter to summarize the present knowledge of the molecular properties of laccase, particularly the relationship between structure and function of these enzymes. Since laccase has been extensively reviewed in the last 10 years, it is not intended to give a full documentation of all the relevant literature. Only a selected number of references will be given here, and the interested reader is referred to a number of reviews.[1-8]

A. Reactions Catalysed

Laccase has a rather low specificity with regard to the reducing substrate. Therefore, a number of quite different substances is readily oxidized. Good substrates are different phenols, both mono-, di-, and polyphenols.[9,10] Of the diphenols, the para-forms are the most readily oxidized ones, but *o*-phenols, e.g., catechol, and *m*-phenols, e.g., resorcinol, are also oxidized at substantial rates. Monophenols inactivate laccase and this is the reason why it was long believed that the enzyme did not oxidize these phenols. It was, however, later demonstrated that monophenols are rapidly oxidized in the presence of gelatin or detergents (Tween® 80), which protect the enzyme from reaction inactivation. Aminophenols and diamines are also good substrates and some inorganic substances like hexacyanoferrate(II) are rapidly oxidized.

B. Biological Distribution and Function of Laccase

There are several reports on the occurrence of laccase in higher plants (for a review see, e.g., Franke[11]). Thus, laccase-like enzymes have been prepared from such diverse plant tissues as peaches and tea leaves.[12,13] However, the most extensive studies of laccase in higher plants are of the enzyme found in various species of the Chinese or Japanese lacquer trees (*Rhus* species). In fact, laccase was first discovered as early as 1883 by Yoshida[14] when he found that the white sap (latex) of these trees was rapidly hardened to a plastic in the presence of air and he postulated the presence of a "diastase" in the lacquer. A few years later Bertrand[15] further purified and analysed this catalyst and named it laccase. He also postulated that laccase is a metalloenzyme as he detected manganese in the protein preparation. About 50 years later Keilin and Mann[16] and Tissières[17] found, however, that the enzyme contained copper, which was essential for the enzymatic function, and that there was no manganese in laccase. The function and properties of some laccases have been studied in great detail during the last decade and will, therefore, be discussed in the following sections.

The main sources for laccase are, however, many different fungi, e.g., the Basidiomycetes *Polyporus*, *Pleurotus*, and *Pholiota* and the Ascomycetes *Neurospora*, *Podospora*, and *Aspergillus*.[18-22] The enzyme is found either in constitutive intracellular forms, as in *Podospora*, or as several inducible exoenzymes in *Polyporus*, *Pleurotus*, *Pholiota*, and *Neurospora*.[20,23] The enzyme is particularly abundant in wood-destroying white-rot fungi which are able to degrade lignin and it is therefore assumed that laccase plays an important role

in lignin degradation (for a recent review, see Kirk[24]). This idea is supported by the absence of laccase in the brown-rot fungi, which are unable to decompose lignin, and also by studies of a laccase-less mutant of the fungus *Sporotrichum*.[25]

Recent studies of the degradation of milled wood lignin by fungal laccase have demonstrated that the substrate is changed on incubation with the enzyme.[26] The M_r of most of the milled wood lignin increases due to polymerization reactions. Only a small part of milled wood lignin is degraded to low-M_r products. It has also been shown that fungal laccase is responsible for demethylation of lignin and lignin-related model compounds and this reaction is thought to be an initial step in lignin biodegradation.[27,28] Another important reaction in the decomposition of lignin by laccase is side-chain elimination.[28,29] Thus, with vanillyl alcohol as substrate both formaldehyde and methanol are liberated by the action of laccase.

It is therefore apparent that laccase plays an important role in lignin degradation. However, other enzymes capable of splitting aryl-alkyl ether linkages must also be involved in lignin biodegradation since laccase cannot split etherified phenols.

Fungal laccase has also been proposed to be involved in the formation of humic acids in the soil.[30] The role of laccase in the *Rhus* species is probably best understood. The enzyme is found in the white latex, which contains phenols (urushiol and laccol). These phenols are oxidized by the enzyme in the presence of dioxygen to radicals, which spontaneously polymerize to a protective structure in similarity with the blood-clotting system in mammals.

II. ENZYME PREPARATION AND CHEMICAL COMPOSITION

A. Isolation of Laccase

As mentioned under Section I laccase is, in many species of fungi, produced both as constitutive enzymes or as inducible exoenzymes. The latter forms are synthesized after the admission of suitable inducers such as different phenolic compounds or ferulic acid.[18-20,31] Of these enzymes, the inducible forms have deserved much recent interest since the yields of the exoenzymes are greatly improved after induction, and the preparations are also simplified, as the formation of other exoenzymes is not influenced or even decreases after induction.[18,20] Only a few recently published methods for the preparation of laccase will be summarized here.

The *Rhus* laccase is obtained from the lacquer which is gathered from a great number of lacquer trees growing in China or Japan. The lacquer is dissolved in cold acetone and the insoluble material is discarded. After evaporating the acetone a greyish powder remains. This powder is dissolved in neutral buffer and the insoluble material is filtered off. To the filtrate, which contains both laccase and stellacyanin and also large amounts of pigments, CM-C50 Sephadex® is added. This cation exchanger binds both proteins strongly but with very little pigment. The final preparation, which includes column chromatography on the same ion exchanger and on DEAE A-50 Sephadex® is therefore greatly simplified.[32,33]

Laccases from the fungi *Polyporus* and *Neurospora* are prepared by somewhat related procedures.[34-37] The organisms are cultivated in steel tanks or in glass containers on minimal media.[34,36,37] After a few days an enzyme inducer is added and the fungi are grown for another few days. Thereafter, the mycelia are filtered off and the filtrate is concentrated either by flash evaporation or by precipitation with ammonium sulfate. The final preparation steps mainly involve column chromatography on the anion-exchanging DEAE A-50 Sephadex® or hydroxyapatite.[34,36,37] In general, the main problem in the preparation of extracellular laccases is to get rid of a great number of different pigments, which sometimes appear to be bound to the proteins or to have the same M_r values or charges as the laccases.[34]

Several chromatographic forms of laccase are obtained from both tree and fungal sources. From *Polyporus* two chromatographic forms, A and B laccase, have been described.[34,35] These fractions can be separated into several isoelectric forms.[38] Thus, fraction A consists

Table 1
M_r VALUE, CARBOHYDRATE AND COPPER CONTENT OF LACCASES

Source	M_r	Carbohydrate content (%)	Copper atoms per molecule	Ref.
Rhus vernicifera	110,000—141,000	45	4—6	32, 41—43
Rhus succedanea	130,000		5—6	42
Polyporus versicolor				
A	64,400	10—14	4	34, 35
B	64,700	10	4	34, 35, 40
Podospora anserina				
I	390,000	24	16	39
II	70,000	25	4	39
III	80,000	23	4	39
Neurospora crassa	64,800	11	3, 4	36, 37
Agaricus bisporus	100,000	15	2	44
Lactarius piperatus	67,000			45
Aspergillus nidulans	85,000			22
Prunus persica	70,000—90,000			12

of two forms, which are isoelectric at pH 3.07 and 3.27. Fraction B contains at least ten components with isoelectric points between 4.64 and 6.76.

B. Relative Molecular Mass and Amino-Acid Composition

The M_r values of some well-defined laccases are summarized in Table 1. Although they range from ≈65,000 to ≈140,000, it is presumed that the main difference in M_r values is due to variations in the carbohydrate content. The amino-acid compositions have been reported for *Rhus vernicifera*, *Polyporus versicolor*, *Neurospora crassa*, and *Podospora anserina* laccases I to III.[32,34,36,37,39] All these enzymes, except *Podospora* laccase I, which is a tetrameric enzyme, probably consist of a single polypeptide chain of about 500 amino-acid residues. The chromatographic forms A and B of *Polyporus* laccase have a different amino-acid composition, which demonstrates that they are coded for by different genes.[34,40] Also, the three forms of laccase produced by the fungus *P. anserina* have a different amino-acid composition.[39]

C. Carbohydrate Content

Both intracellular laccases (*Podospora*) and extracellular enzymes (*Rhus*, *Polyporus*, and *Neurospora*) are reported to be glycoproteins with varying carbohydrate contents (Table 1). The carbohydrate part can, e.g., be as much as 45% of the M_r in *Rhus* laccase, which is also consistent with the high solubility of this enzyme.[32] The amounts of the different carbohydrates are determined for the *Podospora*, *Polyporus*, and *Neurospora* enzymes, but there are no reports on the structure and possible function of the carbohydrate part in any laccase at present.

D. Metal Content and the Three Types of Copper Ions

Today it is generally agreed that laccases from different organisms contain copper, which is essential for the catalytic activity of these enzymes (Section V).

Some early attempts to determine the number and valence of the copper ions in *Rhus* laccase were performed by Nakamura.[41,46] With magnetic susceptibility measurements on the resting enzyme he found that it contained four Cu(II) ions. However, with the then recently introduced EPR technique Blumberg et al.[43] determined that only 70% of the total copper in this enzyme was in the Cu(II) state and that there were six copper ions present. Using the same technique in their studies of the *Polyporus* laccase, Broman et al.[47] found

that the Cu(II) EPR signal corresponded to only ≈50% of the four copper ions present in this protein. Subsequent magnetic susceptibility studies gave similar results, only ≈40% of the copper was in the Cu(II) state in this laccase.[48] The EPR nondetectable copper ions were at that time assumed to be Cu(I) as they showed no paramagnetism even at room temperature.[47,48] As will be shown in Section III, it is presently accepted that these metal ions are in the Cu(II) state in the resting enzyme. The reason why they do not contribute to the magnetic susceptibility is a strong antiferromagnetic coupling between these contiguous Cu(II) ions.

In the early EPR studies of the *Polyporus* enzyme, it was observed that the EPR-detectable fraction of copper showed superimposed signals from Cu(II) in different environments.[49] One of these signals had unusual narrow hyperfine structure not observed in other copper complexes, and it was therefore suggested to arise from native molecules. The other signal had EPR parameters similar to Cu(II) found in denatured molecules and it was at that time suggested to originate from copper bound in destroyed molecules.[47] This idea was strengthened by EPR results of *Rhus* laccase, which exhibited a spectrum with only one signal with narrow hyperfine splitting.[43,50] Later studies with the *Polyporus* enzyme showed, however, that this enzyme contained equimolar amounts of two paramagnetic copper ions in different environments and the concept of three types of copper ions in this laccase was therefore introduced.[51] Many subsequent studies of other laccases have now established that all these enzymes contain three types of copper ions.

There have been various reports on the numbers of metal ions in different laccases (Table 1), e.g., the *Neurospora* enzyme was reported to contain three or four copper ions and the laccases from *R. vernicifera* and *succedanea* were suggested to contain four to six metal ions.[32,41-43] There is, however, general agreement today that laccases contain four copper ions, one each of type 1 and type 2, and two of type 3. One exception is the *Podospora* laccase I, which is a tetrameric protein in which each subunit presumably contains the three types of copper ions in the same relation as in the other laccases.[52] The other exception is the laccase from *Agaricus bisporus* which is not blue and contains only two copper ions.[44]

E. Reversible Removal of Copper

The metal ions are firmly bound in laccase and are, e.g., not removed by dialysing the enzyme against EDTA at neutral pH.[53] The metal ions can, however, be reversibly taken out if the enzyme is dialysed against cyanide at pH 7 to 8. This method has been used in several studies of the *Rhus* laccase, where most of the copper ions have been removed.[17,53-56] When the metals are removed the enzyme loses activity, blue color, and the absorption band at 330 nm. These absorption bands are associated with type-1 and type-3 copper ions (see Section III.A). The original properties of the native proteins are restored if the apoenzymes are treated with Cu(I) but not with Cu(II),[50] but cf. Tissières.[57]

Although these results demonstrate that copper is essential for the enzymatic activity, they do not show if all three types of copper ions present in laccase are necessary for activity since there was no selective removal of only one type of copper. Later studies of the *Polyporus* and *Rhus* laccases report that it is possible to reversibly remove only the type-2 copper.[33,57,58] Both type-2 copper-depleted (T2D) enzymes are devoid of activity which demonstrates that this type of copper is an integral part of the protein [and not just a "denatured" Cu(II), cf. Section II.D] and therefore has a function in the catalytic reaction. How this metal is involved in the reduction and reoxidation mechanism has recently been studied with the T2D *Rhus* enzyme and the results will be discussed in Section V.[33]

F. Structural Studies

Only very limited information about the structure of laccases is available today. No crystals suitable for X-ray analysis have yet been produced despite serious attempts by several expert

groups in metalloprotein crystallography. The difficulties to obtain well-ordered crystals probably depend on the rather high carbohydrate contents of these proteins (Table 1), and the removal of some of this carbohydrate might be necessary for a successful crystallization. There appears to be only one group actively working on the amino-acid sequence of a laccase at present and only very few data have been reported.[40,59]

The complete amino-acid sequence has been determined for a number of other blue proteins such as azurins (Az's) and plastocyanins (Pc's). They all contain a type-1 copper ion and since the three-dimensional structure has been determined for one Pc and one Az, it is known that the type-1 copper in these proteins is coordinated to two-histidine, one-methionine, and one-cysteine residues in a distorted tetrahedral geometry.[60,61] A statistical analysis of all sequences known for blue proteins has recently been made and the results suggest that the blue copper proteins are related and therefore derived from a common ancestor gene.[62,63]

The amino-acid sequences have also been determined of two large fragments of human ceruloplasmin covering over half of the primary structure of this oxidase.[64] The two large fragments show a very pronounced sequence homology around a cysteine residue with the small blue proteins (Figure 1). It is therefore proposed that the two type-1 copper ions in ceruloplasmin are bound in a similar way as in the small blue proteins.[64,65]

A limited sequence around the single cysteine residue has also been determined in *Polyporus* laccase, and a comparison with the corresponding sequences in Az, Pc, stellacyanin (St), umecyanin,[66] and the two ceruloplasmin fragments is shown in Figure 1. Included are also a part of the reported sequences of polypeptide II in yeast,[67] human, and bovine cytochrome *c* oxidase. These peptides show remarkable homologies with the blue proteins, and cytochrome *c* oxidase would therefore contain a type-1 copper site.

The 19,000-M_r fragment of ceruloplasmin and the laccase peptide have histidine residues on both sides of the free cysteine residue. This structure is absent in the other proteins and in the 50,000-M_r fragment in ceruloplasmin. Since ceruloplasmin contains two type-1 copper sites but only one type-2 copper ion, it was suggested that these histidine residues are ligands to the single type-2 copper in both ceruloplasmin and laccase.[59] This idea is also supported by kinetic studies on laccase which suggest that there is a conformational coupling between these metal ions (cf. Section V).[68]

Although there appears to be strong homology between the different proteins, as concerns the proposed type-1 copper binding sites, it is apparent that this metal cannot be coordinated in identical structures in all blue proteins; e.g., St lacks methionine which is a metal ligand in Az and Pc and probably also in ceruloplasmin and cytochrome *c* oxidase (Figure 1).[69] Furthermore, there are two reports suggesting that cysteine is not a ligand to type-1 copper in two different proteins. Thus, this amino acid is absent in the small blue copper protein called plantacyanin and the single free cysteine residue in the *Rhus* laccase can be modified without changes in the binding of the type-1 copper or of the enzymatic activity.[70,71] A new Cu(II) EPR signal has been observed in two laccases and in cytochrome *c* oxidase.[33,72,73] These signals were shown to originate from the earlier EPR silent type-3 copper ions in laccase and the Cu_B in cytochrome *c* oxidase. Similar rhombic Cu(II) EPR signals have also been found in other proteins containing binuclear centers such as Cu/Zn-superoxide dismutase (SOD) and half-methemocyanin (Table 5).[74,75] Of these proteins the metal coordination is known only for Cu/Zn-SOD. Crystallographic studies of this protein have shown that its copper ion is bound to the three histidines, which are encircled in Figure 2, and to a fourth histidine farther away in the sequence.[76,77] The similarity between the rhombic Cu(II) EPR signals in the different proteins led to the proposal that these copper ions might be coordinated in somewhat similar ways.[72] This suggestion gains support from the reported sequences of cytochrome *c* oxidase polypeptide II and ceruloplasmin given in Figure 2 since they show great homology with the copper-binding site in Cu/Zn-SOD. It is therefore possible that at least one of the type-3 copper ions in the blue oxidases is bound in this peptide sequence

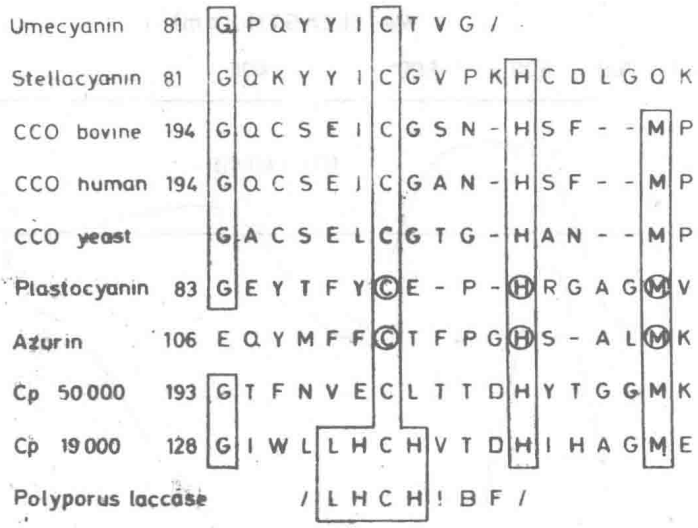

FIGURE 1. Sequence homology in some blue copper proteins and in subunit II of cytochrome *c* oxidase (CCO). Two large fragments with M_r of 19,000 and 50,000 have been sequenced in ceruloplasmin (Cp). (After Dwulet, F. E. and Putnam, F. W., *Proc. Natl. Acad. Sci. U.S.A.*, 78, 2805, 1981. With permission.)

FIGURE 2. Sequence homology in copper-containing oxidases: Cu/Zn-superoxide dismutase (SOD), ceruloplasmin (Cp), and subunit II of cytochrome *c* oxidase (CCO). (After Dwulet, F. E. and Putnam, F. W., *Proc. Natl. Acad. Sci. U.S.A.*, 78, 2805, 1981. With permission.)

and that the earlier EPR invisible Cu_B in cytochrome *c* oxidase has a type-3 copper character instead of the proposed St-like properties.[78]

III. SPECTROSCOPIC AND MAGNETIC PROPERTIES

As discussed in Section II, there is only very limited information about the structure of blue oxidases available at present. However, the unusual spectral properties of the blue proteins and oxidases have attracted much interest from various spectroscopists and many attempts have been made to explain the unusual stereochemical structure of the type-1 copper site.

Fortunately, the three-dimensional structures of two small copper proteins have recently been elucidated. With this important information about the coordination of their type-1 copper and the results from extensive studies of spectroscopic and magnetic properties,

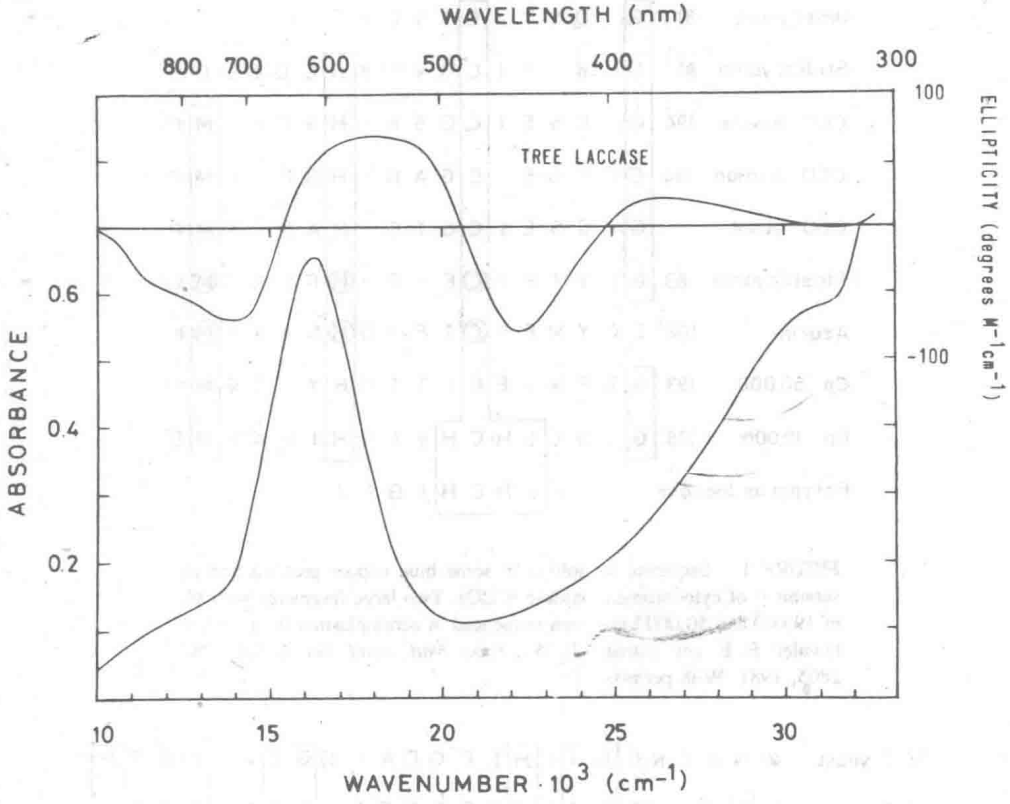

FIGURE 3. Optical absorption and circular dichroic spectra of *Rhus* laccase. (Taken from Falk, K.-E. and Reinhammar, B., *Biochim. Biophys. Acta*, 285, 84, 1972. With permission.)

which are summarized in this section, several detailed structural models have been suggested for the other blue proteins.

A. Optical Spectra

The optical absorption and circular dichroism (CD) spectra of laccases (and ceruloplasmin) are all very similar. As an example, Figure 3 shows these spectra of the *Rhus* enzyme. The absorption spectra of these oxidases (and of all other blue proteins such as Az and St) are dominated by a strong band at ≃600 nm which is responsible for the beautiful blue color of these proteins. The molar absorption coefficient per type-1 Cu(II) of this band is ≃4,000 to 6,000 $M^{-1}cm^{-1}$ in the different proteins and this value is about two orders of magnitude larger than for other Cu(II) complexes.[2] Two weaker bands at ≃800 and 450 nm, which stem from the same chromophore, the type-1 Cu(II) site, are not always resolved in the blue oxidases but are revealed in the simpler blue proteins, Pc and St.[79,80] These absorption bands and the other transitions in the CD spectrum have all been assigned to the type-1 Cu(II). This can best be shown with the small proteins which contain only the type-1 copper; e.g., on reduction of this Cu(II) in St all the optical transitions between 33,000 and 5,000 cm^{-1} vanish.[80,81] This Cu(II) can also be selectively reduced in the *Polyporus* laccase leaving the other types of copper oxidized. The CD spectrum of this partly reduced enzyme lacks all the transitions which are present in the oxidized enzyme and this result shows that the other types of copper ions do not contribute to the CD spectrum of this enzyme.[81]

In addition to the transitions shown in Figure 3 two more bands have recently been detected below 10,000 cm^{-1} in the *Rhus* laccase.[82] Table 2 shows the positions and signs