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POLYPHENOL OXIDASES IN PLANTS—RECENT PROGRESS

ALFRED M. MAYER

Department of Botany, Hebrew University of Jerusalem, Jerusalem, Israel

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Key Word Index—Higher plants; polyphenol oxidase; catechol oxidase; laccase; properties; structure; reaction mechanism; physiological function.

Abstract—Progress in the plant polyphenol oxidases in the period 1978–1986 is summarized. Methodology, occurrence, properties and physiological function of laccases and catechol oxidases are critically reviewed. The advances in the understanding of reaction mechanisms are cited. The plant polyphenoloxidases remain enzymes in search of a function.

INTRODUCTION

Since we last reviewed the plant polyphenol oxidases [1] the international nomenclature has again been changed, monophenol monooxygenase (tyrosinase) being referred to as 1.14.18.1, diphenol oxidase (catechol oxidase, diphenol oxygen oxidoreductase) as 1.10.3.2, and laccase as 1.10.3.1. This new classification is no improvement since it differentiates between two reactions of the same enzyme, formerly referred to as cresolase and catecholase activities. At the same time the clearly distinct laccases from fungi and higher plants are still given the same number. We will therefore retain the general terms of catechol oxidase and laccase for the sake of simplicity.

A large number of papers have been published since the last attempt to review the topic extensively and it is inevitable that we will be selective in our coverage; yet an attempt will be made to deal with various aspects of polyphenol oxidase biochemistry and physiology. A number of reviews covering certain aspects have appeared in recent years. Laccase has been reviewed with particular stress on the properties of the enzyme and the nature of the catalytic site [2–6]. These bring surveys of the reaction mechanism up to date almost to the time of writing of this review. Catechol oxidase has also been reviewed, but less comprehensively. Its reaction mechanism has been considered [7–9] and the molecular properties of tyrosinase reviewed [10, 11] and attention given to its overall biological function and subcellular location [12, 13]. As will be seen in the following sections, progress has been impressive in unravelling the basic structure of several of the polyphenol oxidases and in understanding some of the basic mechanisms of the oxidation–reduction process. However, in the understanding of the biological function, progress has been very slow and this problem remains essentially unsolved.

METHODOLOGY USED IN ASSAY AND STUDY OF ENZYME PROPERTIES

In the determination of laccase, syringaldazine has been successfully used for the quantitative assay of the fungal enzyme [14–16], thus extending the use as first described [17]. The use of 2,6-dimethoxyphenol as a laccase substrate is also worth mentioning [18]. The number of substrates reported to be attacked by laccase seems to increase steadily. The location of both laccase and catechol oxidase by electron microscopy using specific substrates has been attempted. These have located catechol oxidase quite definitely in plastid thylakoids [19–21], in accord with previous reports [1]. In the case of laccase definite assignment of its location using the EM is uncertain because its location is based on the assumption that failure to oxidize tyrosine and the ability to oxidize DOPA can be regarded as proof of laccase activity and precludes catechol oxidase activity [22]. The cytochemical localization of catechol oxidase outside plastids (e.g. in fungal cells) and of laccase still presents serious problems.

HPLC has been used to follow enzyme activity and is obviously useful in order to separate the products of enzyme activity, many of which arise by secondary reactions [23–25]. Some success has been obtained using antibodies raised against either laccase [26, 27] or catechol oxidase [28–30].

Most of the purifications recently reported for catechol oxidase follow known procedures, but hydrophobic gel chromatography seems to have distinct advantages [31, 32], and its use should be extended. The separation of enzymes by electrophoresis is now a standard procedure. Improvements have been reported by purification of the enzyme in the presence of inhibitors of proteolysis which reduces the numbers of apparent isozymes on the gel [33]. Electroblotting of catechol oxidase [34] and SDS tech-

niques [35] have also been successfully applied. Inhibitors continue to be used extensively in studies of the physiological function of both laccase and catechol oxidase. The two activities can be differentiated by the use of cinnamic acid derivatives to inhibit catechol oxidase and cationic detergents such as CTAB to inhibit laccase [36]. Tropolone acting as a Cu^{2+} chelator is very effective in inhibiting catechol oxidase, at concentrations in the μM range [37]. Its effect on laccase has not yet been reported. Often inhibitors are used in order to establish the function of enzymes. It cannot be stressed too strongly that inhibitor studies alone cannot prove the participation of an enzyme in a physiological process, a fact often ignored.

An interesting if somewhat exotic use for laccase has been suggested. A laccase membrane electrode has been constructed and has been used to measure epinephrine using a quinol–quinone couple [38]. Laccase has also been used to measure glucosidase or amylase activity, by coupling oxidation of a phenol and 2,6-dibromo-4-aminophenol [39]. The method was based on the release of a sugar from a phenolic glycoside followed by the enzymic oxidation of the phenol released. The possible uses of laccase or catechol oxidase for analytical studies on the one hand or the immobilization of the enzymes in order to use them to carry out controlled oxidation reactions continued to arouse interest, although until now success has been rather limited.

The most impressive use of modern methodology is in the analysis of the reaction mechanism of laccase and catechol oxidase. The full array of biophysical techniques has been applied to this problem. Thus EPR [40, 41], circular dichroism measurements [42], Raman spectroscopy [43] and electron nuclear resonance spectroscopy [44, 45] have all been used in recent work on laccase. A large number of studies have utilized the technique of partial copper depletion of laccase [46, 47] including pulse radiolysis [48], laser radiation of the depleted enzyme [49] and X-ray absorption studies [50]. Metal substitution by, for example, Hg^{2+} [51] or removal and resubstitution of Cu^{2+} [52] have been used to distinguish between some of the different copper sites of laccase. Another approach has been the titration of the copper sites using reagents such as azide [53], nitrite [54] or nitric oxide [55]. Stopped flow kinetics have been used to follow the catalytic reaction sequence of tree laccase [56]. The results obtained using these methods will be discussed in the section on structure and reaction mechanism.

There have been fewer reports on catechol oxidase using these techniques, because of the known EPR silence of copper in the enzyme and because very few really pure preparations of this enzyme are available. The assay of competitive binding of inhibitor has been used to study *Neurospora* catechol oxidase (tyrosinase) [57, 58].

OCCURRENCE OF LACCASE AND CATECHOL OXIDASE

Reports continue to appear which support the widespread occurrence of these enzymes and which indicate the almost universal appearance of catechol oxidase. Only a few of the more recent reports will be cited. Almost every basidiomycete examined appears to contain laccase, extra- and/or intracellular, at least at some stage of its development. Among the fungal laccases recently described, that of *Stereum* has been partially purified and characterized [59]. Extracellular laccase appears to be present in many mycorrhizal fungi [60]. In this respect the observation that most ectomycorrhizal fungi do not produce catechol oxidase is perhaps significant [61]. The species *Lactarius* is an exception to this generalization and further investigations are therefore needed. Other reports do indicate a quite general presence of catechol oxidase in mycorrhiza [62]. Both laccase and catechol oxidase are sufficiently stable that they can survive in the decomposing plant residues in soils. In this state they may even become part of the humus complex [63–65].

An extremely new interesting laccase has been shown to be excreted into the growth medium of cells of *Acer pseudoplatanum* (sycamore) in suspension cultures [66]. This enzyme is of considerable interest as providing an additional rare example of a higher plant laccase. It is secreted in quite large amounts and appears to be a typical glycoprotein laccase with a very high carbohydrate content. The carbohydrate moiety of this enzyme has been investigated and fully characterized. It has been shown to be an *N*-linked oligosaccharide with a recurrent xylose-containing structural unit [67]. The oligosaccharides in it are of the biantennary complex type. It contains xylose–mannose and fucose-*N*-acetylglucosamine linkages.

An intriguing enzyme, described as a phenoloxidase, has been shown to be present in the pathogenic fungus *Cryptococcus neoformans*, which can cause meningitis [68–70]. The presence of the enzyme appears to correlate with the virulence of the pathogen, as shown by experiments using mutants. Although the enzyme oxidizes phenols [68], it seems to be debatable whether it is a catechol oxidase, since its substrate specificity and inhibitor response differ considerably from those normally associated with catechol oxidase. Further characterization is needed in this case.

There are far more reports on catechol oxidase both in fungi and plants. Interest in wheat catechol oxidase has revived because of its possible significance in baking [71–73], since its presence may cause darkening in dough.

The attempt to further the cultivation of yam has led to an investigation of the properties, distribution and amount of catechol oxidase in various yams, e.g. *Dioscorea* sp. [74–76] as well as in *Xanthosoma* [77]. The yam enzyme appears to be a typical catechol oxidase with all the characteristics of these enzymes. The catechol oxidases in grapes continues to arouse interest because of its apparent contribution to the quality of wines [78–80]. Tissue culture of plants for secondary product formation continues to be a topic which is extensively researched. Catechol oxidases have been investigated in suspension cultures of tobacco [21, 81] and of *Mucuna*, which has not been previously studied [25, 82]. Some other species whose catechol oxidases have not been previously described are okra seeds (*Abelmoschus*) [83] and guava (*Psidium*) fruit [84]. Enzyme activity has been followed in date, *Phoenix dactylifera*, fruit [85]. Perhaps not surprisingly, galls formed in a number of plants appear to contain elevated levels of catechol oxidase as compared to normal, non-infected tissue [87–89]. Since gall formation may be regarded as a kind of wound reaction, this observation is therefore in line with reports on tissue response to injury.

LOCATION AND CONTROL OF ENZYME ACTIVITY *IN VIVO*

Laccase

The site at which the enzymes are present continues to raise a variety of problems. In the case of laccase this is closely linked with the question of its function during

development. Whether laccase is specifically formed during fruiting of various fungi is still not clear. The evidence is quite contradictory. An extracellular laccase from *Lentinus* appeared in fruiting bodies; the increase in enzyme activity was associated with the rapid growth of non-pigmented mycelium and the formation of pigmented primordia [90]. In *Aspergillus parasiticus* laccase was found only in conidiating cultures, but laccase formation and conidiation did not seem to be correlated. The level of enzyme production was partially controlled by the composition of the growth medium—$(NH_4)_2SO_4$ suppressing and glutamate promoting laccase formation [91].

The laccase of *A. nidulans* has also been studied and in rather great detail. This laccase was present in conidia and showed all the typical characteristics of fungal laccases. Laccase isolated from white spore and green spore strains differed somewhat in characteristics, but the differences arose after pigmentation had occurred. The difference in colour of the strains may have been due to lack of substrate rather than due to differences in their laccase or its amount [92]. Further studies of this fungus showed that two distinct laccases occur which differ immunologically and electrophoretically. One was associated with conidial pigment formation and the other with cleistotheca formation. Cytochemical methods to definitely localize the enzyme were not entirely convincing, because the reagent used also reacts with catechol oxidase [93].

In *Coprinus* a somewhat similar situation was observed. A clear correlation between localized laccase formation and the development of primordia for carpophores was observed, but laccase was not apparently involved in the actual developmental process, nor was there a critical level required for differentiation [94]. In *Agaricus* an extracellular laccase is produced, which has been fully characterized. Antibodies have been raised against it [95]. In this case there seemed to be no induction of enzyme formation by components of the culture medium. The enzyme closely resembled other fungal laccases in molecular properties. During fruiting body development the level of this extracellular laccase decreased due to both inactivation and proteolysis, making a function in fruiting body formation very unlikely [96].

In *Podospora anserina*, laccase actually formed during cell lysis and it was demonstrated that this was a developmentally regulated process, involving *de novo* synthesis. Here, therefore, extracellular enzyme formation is correlated with lysis and its control [97]. The induction of laccase formation in various fungi has been documented in many cases. The inducers can be quite different. In *Polyporus* resorcinol acted as inducer [98]. During the testing of induction of a variety of laccases, it was noted that while 2,5-xylidine induced extracellular laccase formation in *Fomes*, *Pholiota*, *Trametes* and *Pleurotus* it failed to do so in *Botrytis cinerea* and *Rhizoctonia* [99]. Induction of laccase formation in *Botrytis* has been studied in detail in our laboratory [100–104]. This work showed a very complex situation with regard to the control of the induction of this enzyme in *Botrytis*. The enzyme is produced in very small amounts or is absent entirely in the absence of inducer. In the presence of inducer it is formed, but its molecular properties such as MW, electrophoretic behaviour, substrate specificity, heat inactivation and isoelectric point all change depending on the nature of the inducer. Among the effective inducers were gallic acid, coumaric acid and grape juice. Not only did the properties change, the amino acid and sugar composition of the enzyme also changed depending on the inducer. In the presence of two different inducers, two distinct enzymes formed, indicating that different genes are activated by different inducers. Induction is developmental in nature; the inducer must be present long before the enzyme is actually produced in quantity. Very small amounts of laccase may be present constitutively. Most interesting, the actual amount of laccase produced in the medium was regulated by a second inducer, which was pectin or a derivative or breakdown product of it [104]. These findings make it clear that it will be quite difficult to establish unequivocally the developmental role of laccase in fungi. The lack, at present, of a really satisfactory method of localizing it cytochemically or under the EM adds to this difficulty. Perhaps immunological techniques linked to electron microscopy will be able to resolve at least this question.

Fungal catechol oxidase

The occurrence of catechol oxidase seems to be correlated with the appearance of fruiting bodies in a number of basidiomycetes [105], but there does not appear to be any causal relationship. The isozyme pattern of catechol oxidase extracted from basidiomycetes at different stages of their development also shows no important differences [106]. A novel highly effective inducer of catechol oxidase has been found in the case of *Coprinus* [107]. Enzyme formation was induced by 2-deoxy-D-glucose, but fruiting body formation was inhibited by it. Nevertheless, regulation of the two processes was distinct and no evidence for combined regulation was found. Again the correlations seemed to be fortuitous. In *Neurospora* a low molecular weight inhibitor accumulated, but this is prevented if the sulphate ion supply is limited [108]. The mechanism of regulation appeared to be that factors causing a reduction of the inhibitory –SH compound, such as osmotic stress or phosphate starvation, derepress catechol oxidase formation. The complicated fashion in which the amount of catechol oxidase in fungi may be regulated is illustrated by the situation in *Aspergillus oryzae* [109]. In this species a protyrosinase (procatechol oxidase) has been found, which can be activated by exposure to pH 3.0. The pro- and active forms of the enzyme differ in their protein conformation. In addition, as isolated, the proenzyme appeared to be firmly associated with proteinases. The activity of these proteinases was responsible for the multiplicity of bands observed following electrophoresis of the enzyme. Rapid acid acitivation is strongly reminiscent of the behaviour of grape catechol oxidase [110]. The association of a proteinase with the enzyme throughout purification should serve as a warning to all who do not pay sufficient attention to this point and may well be a common phenomenon [see also 33].

Plant catechol oxidases

That catechol oxidases are present in the chloroplasts of plants is by now fairly widely accepted [13, 19–21]. Differences have been reported in the catechol oxidase content of bundle sheath or mesophyll cell of *Sorghum*, it being absent from the former [111]. Its location within the thylakoids has also been confirmed in *Aegopodium* [112]. Further evidence of the chloroplastic nature of catechol oxidase comes from work with the enzyme from *Vicia faba* [113]. Poly A mRNA was translated *in vitro* and the enzyme formed was identified by immunoprecipi-

tation and shown to have an M_r of 45 000. This is identical with the native enzyme. Moreover it appears that the enzyme was transported into the chloroplast without processing, an unusual and important finding, if confirmed. Almost all reports on the transport of protein into chloroplasts indicate that they are processed during passage into the chloroplast. It may be noted that evidence was brought in this work that the *Vicia faba* enzyme contains a small amount (3–4%) of carbohydrate. From work with *Nicotiana* [114] it is quite clear that the chloroplastic catechol oxidase is nuclear coded, and supporting evidence comes from the work on *Vicia* and *Aegopodium* [112, 113].

The highly convincing evidence for the chloroplastic location of catechol oxidase has been challenged by work on catechol oxidase in carrot cultures [115–117]. A procatechol oxidase was isolated, which was activated by Ca^{2+} or Mg^{2+} at 1 mM and also by trypsin. The authors claim that this enzyme is soluble in nature and becomes activated and then associates with various subcellular fractions thus accounting for the appearance of the enzyme in different organelles, including perhaps chloroplasts. Although these findings might account for the widespread reports of the association of catechol oxidase with organelles [1], it is difficult to accept that it accounts for its presence in chloroplasts. The evidence for the presence of the enzyme in thylakoids seems to be compelling [1, 12, 13, 19–21]. In addition, the work on carrot cell cultures addresses the question of the time of appearance of catechol oxidase. It appears that it is restricted to embryogenic cultures only and might serve as a marker for a finite developmental stage. Indirect support for this view comes from the finding that α-amanitin is oxidized only by differentiating carrot cells and that this oxidation is mediated by catechol oxidase [118]. However, although there is an indication of developmental control of catechol oxidase appearance, studies using *in vitro* translation of mRNA indicated that in *Vicia* at least it is possible to get translation products corresponding to catechol oxidase at many stages of development [119].

Since catechol oxidase is often activated in plant tissues the factors responsible for activation have been studied. In *Vicia faba* activation of the latent catechol oxidase is obtained either by acidification or release of a free fatty acid [120]. In spinach the inactive catechol oxidase can be isolated from thylakoids and linolenic acid can bring about 100% activation [121]. This suggests that indeed natural activators of catechol oxidase do occur. This might be related to the often observed activation of latent catechol oxidase during senescence, which has been ascribed to release of the enzyme from binding to the thylakoids [122, 123]. Senescence may well involve release of fatty acids during membrane breakdown. Natural inhibitors of catechol oxidase have also been described a number of times. Among more recent partially identified ones are oxalate in spinach leaves [124] and quercetin and leucoanthocyanin in tea leaves [125]. Although the latter inhibitors were said to be active at quite low concentrations in no case has it been convincingly shown that such inhibitors regulate enzyme activity *in vivo*.

Stress conditions can also affect catechol oxidase activity. Salt stress caused increases in enzyme activity in wheat and barley with differences in the response of tolerant and resistant varieties, increases being greatest in the salt sensitive ones [126]. Mineral nutrition such as Ca or P deficiency [127] seemed to result in decreased enzyme activity and boron deficiency appeared to result in increases in its activity in dicotyledons, but not in monocotyledons such as *Sorghum* [128]. These results should all be considered together as showing that interference in the normal metabolism of the plant results in a multitude of responses including changes in the level of catechol oxidase activity. No really specific effects have been demonstrated. In the same light the effect of ventilation during storage of avocado must be analysed. Poor ventilation during storage or water stress during growth resulted in elevated levels of enzyme, apparently due to release from membranes [129].

Since copper is an integral part of catechol oxidase, attempts to relate copper supply and enzyme activity have been made. Copper deficiency reduces enzyme activity in subterranean clover in its aerial parts, except in very old parts [130], as is also the case for *Chrysanthemum* [131]. More detailed analyses in clover attempted to resolve the question of when the enzyme is actually synthesized [132]. Leaves of plants grown under conditions of copper deficiency failed to develop enzyme activity even if Cu^{2+} was supplied at a later stage. Apparently the holoenzyme can only be formed during an early developmental stage. It is not yet clear whether the apoenzyme can be formed and fails to incorporate copper or whether it too is not formed during copper deficiency and its subsequent formation later in development is repressed [115–119]. The difference in pattern of catechol oxidases between organized and unorganized tissues of *Solanum melongena* was said to reflect changes of gene activity, which occurred during development [133]. The question of the stage at which catechol oxidase is formed in a given tissue appears to be central when trying to understand the control of its activity in the plant. The overall evidence is still full of contradictions which are difficult to reconcile and insufficient data are available. The initiation of fibre formation in cotton can be regarded as an early or late stage of development, depending on which plant organ is being considered, the plant or the fibre. In cotton fibres, formation is associated with an initial increase in enzyme activity [134]. Does this support the idea of its formation early in development?

Changes in catechol oxidase activity in suspension cell cultures have been studied using ethionine or norleucine as specific developmental inhibitors of enzyme formation [21, 81, 82]. In cultures of *Mucuna pruriens* catechol oxidase activity rose during 8 days of culture, the rise being affected by the level of nitrogen in the basal medium. Enzyme activity peaked just before cell growth reached its maximum [82]. Very low levels of ethionine added to the growth medium (100–1000 μM) induced considerable increases in enzyme activity, in contrast to earlier reports which showed that higher concentrations repressed activity [135]. The difference between the effect of this methionine antagonist may be due to differences in methionine/inhibitor ratios in the culture medium. The use of a different methionine antagonist, norleucine, provided further evidence that enzyme activity can be regulated by manipulating the ratio of antagonist to amino acid [81]. It also provided convincing proof that enzyme activity can be repressed without in any way effecting the viability or growth rate of the tobacco cultures. The only structural change induced by norleucine was appearance of amyloplasts instead of plastids. Whether this should be regarded as a regulatory mechanism seems doubtful, since it could be shown that enzyme

with reduced activity was synthesized into which the amino acid antagonist had been incorporated. These results do not therefore answer the question whether or not developmental events are among those controlling enzyme synthesis in the plant. They also do not provide an unequivocal answer to the problems about location with the tissue, which we discussed above.

PHYSIOLOGICAL FUNCTION OF POLYPHENOL OXIDASES

Laccase

Considerable attention has been focussed on the function of the extracellular laccase in the decomposition of lignin. The problem has been approached on the one hand by studying the possible mechanism by which laccase causes lignin breakdown and on the other hand by attempts to inhibit laccase or reduce its level in wood rotting fungi. In addition to the well recognized oxidative activity it is clear now that laccase also can demethylate methoxy groups [136]. The mechanism of demethylation has been studied in some detail [137, 138] and indirect evidence indicates that this function is important in lignin degradation. This degradative activity is closely associated with the ability of laccase to affect the degree of polymerization of the substrate. High molecular weight lignosulphates were decomposed while low molecular substrates were polymerized [139]. The presence of lignosulphates in the growth medium stimulated laccase production [140] and mutants of *Pleurotus* lacking laccase also degraded lignin poorly [141, 142]. The delignification of cotton straw required the preliminary action of laccase [141]. The wood decaying ability of *Heterobasidium* (= *Fomes*) was shown to be strongly oxygen dependent, and this indicated a K_m O_2 of the fungal laccase of 10-20%. However, wood degradation was not absolutely dependent on O_2 [143]. In contrast to the results showing a function of laccase in wood decomposition, in *Daedalea* there was no correlation between fungal mass and laccase production. Additional evidence against the function in lignin breakdown is also reported. Mutants of *Phanerochaete* which lacked the ability to oxidize *o*-anisidine was still able to degrade lignin, but it was not shown that the ability to oxidize *o*-anisidine is characteristic only of laccase [145]. Antibodies raised against *Coriolus* laccase were used to inhibit the enzyme but the fungus was nevertheless able to degrade lignin [27]. Similarly in the case of *Fomes* inhibition of laccase did not prevent lignin degradation [146]. These apparent contradictions may be related to factors controlling laccase formation. The enzyme is partly constitutive and in part produced only in the presence of suitable inducers (see above).

The role of phenolic compounds in controlling laccase formation has been studied in *Heterobasidium* and in *Fomes*. Phenols added to *Fomes* in liquid culture inhibited its growth [147]. The inhibitory phenols could be oxidized by the fungal laccase. Moreover the same phenols when added to spruce wood failed to inhibit fungal growth. The phenols were able to induce laccase formation. Exposure of *Heterobasidium* to lignosulphate induced *de novo* formation of an extracellular laccase. The hyphae appeared to contain specific receptor sites for phenolic inducers. These sites could be blocked by synthetic macromolecules which were not degraded by the fungus. As a result, induction of laccase formation by phenols was blocked [148]. The apparent multiple action of laccase in lignin degradation must also be taken into account. Laccase can oxidize, demethylate, polymerize or depolymerize. Thus it is possible that its importance lies not in lignin oxidation *per se*, which perhaps can be affected in other ways, but in its ability to polymerize oxidation products of lignin which arise during lignin oxidation.

A genetic analysis of laccase formation in *Pleurotus* is the only detailed study of its kind [149]. Multiple laccases were found; there was a high degree of genetic variation in isolates of wild strains of *Pleurotus*, one isozyme being specifically associated with fruiting body formation. Both sympatric and allopatric speciation occurs in basidiomycetes as determined in this investigation. Thus when considering the function of laccase in a specific physiological process it will be necessary to identify which of the multiple forms is studied and to ensure, when making comparisons, that a well defined species of the organism under investigation is used.

The possibility that laccase is involved in the infection process during pathogenesis has also been considered. *Rigidoporus* infects the roots of the rubber tree, *Hevea*. Laccase activity in the roots showed a clear gradient with high activity in the infected tissue, near the progress of the fungal infection, while it was low in old infected tissue. Healthy tissue was much better able to induce laccase formation than old infected tissue [150].

Similar findings have been brought with regard to the infection of cucumber by *Botrytis* [104]. In this work it was found that laccase activity showed a gradient from the infected area to the edge of the uninfected region of the fruit. It was hypothesized that the laccase is involved in the initial process of infection. Perhaps it overcomes some of the defence reactions of the host plant by oxidizing and polymerizing endogenous plant phenols, thereby rendering them non-toxic. Although the results are suggestive that the above mechanisms are operating, many more experiments will be needed before the hypothesis can be confirmed.

Fungal and plant catechol oxidase

Most of the recent papers have pursued the idea that in some way catechol oxidase is involved in fungal and plant interactions. Research has attempted to show correlations between levels of enzyme activity or location and the degree of infection which is achieved. Results are very difficult to interpret. Lack of correlation between level of enzyme and localized necrosis has been reported [151-153], but in other cases correlation between infection and catechol oxidase has been reported to exist [154]. It has been claimed that catechol oxidase activity in the host actually drops as a result of infection [155]. Fungal catechol oxidase can cause oxidative polymerization of host phenolics, making them less effective [156]. This recalls similar roles assigned to laccase. Overall correlations between catechol oxidase level and spread of infection are probably not very relevant, since even a very localized increase of host enzyme activity at the edge of the infection might limit spread [157], while the very localized production of fungal enzyme activity might suffice to overcome a host reaction. The results of catechol oxidase action might be very indirect. As already shown in the past products of the oxidative activity of catechol oxidase can inhibit certain fungal enzymes, such as endo-

polygalacturonases [158]. Coupled oxidation reactions can inactivate alkaloids, for example in the capsule of *Papaver* [159]. This may affect infective processes, if the alkaloids have a protective function, which is by no means certain. The ability of fungal catechol oxidase to oxidize tyrosine residues of proteins to DOPA followed by dopaquinone formation [160] constitutes an important addition to the known activities of the enzyme. Oxidation of a protein might lead to inactivation, and therefore this activity of the enzyme might be related to its function, a point which may be worthwhile pursuing.

Catechol oxidase activity in *Sorghum* during grain development decreased while at the same time appreciable amounts of tannins were formed. This led to the suggestion that the initial protective role of the enzyme was being taken over by the tannin [161]. Although this suggestion is interesting, at this stage it still must be regarded as speculative.

Renewed attempts have been made to relate levels of growth substances with enzyme activity. As in the past the results have been negative or unconvincing. Changes in the grafting ability of peach shoot apices, which show seasonal variations, were not correlated with catechol oxidase activity [162]. The ability of apple cuttings to root did correlate with increases of catechol oxidase activity in the xylem sap. Oxidation of phloridzin by the enzyme resulted in the formation of compounds with some activity of induction of rooting of the cuttings [163], but it seems at least questionable that this is the cause of variability in the ability to root. Transition of plants to the flowering stage was found to be correlated with enzyme activity [164], as have levels of IAA [165]. Such correlative studies are at best the basis for continuing experiments. At present they are not sufficiently convincing to assign a definite function to catechol oxidase.

Functions have also been sought at the biosynthetic level. The enzyme from *Agaricus* catalyses the formation of 2-hydroxy-4-iminoquinone, which is an SH reagent. Steps in the reaction have been identified. Since the quinone is metabolically active, this constitutes the first demonstration of the involvement of catechol oxidase in a definite biosynthetic pathway [166]. A *p*-coumaric acid hydroxylase from spinach has been described. This is a Cu enzyme, apparently a catechol oxidase, whose activity was regulated by light, as far as its hydroxylase activity was concerned [167]. Whether it is a genuine catechol oxidase and whether it actually functions *in vivo* in the hydroxylation of *p*-coumaric acid remains to be established, since a family of non-copper hydroxylases is known to exist [9]. Hydroxylation of carbon 3′ of compounds such as kaempferol has been shown to be associated with catechol oxidase from *Zea* and its possible significance in flavonoid biosynthesis has been discussed [168]. However, tissues in which catechol oxidase activity in the chloroplasts was repressed by the use of tentoxin, which causes the loss of plastidic enzyme, contained a normal complement of flavonoids [170]. Consequently the involvement of the enzyme in the formation of phenolics is at best open to debate. The apparent absence of hydroxylative activity in many enzyme preparations is probably at least partly the result of methods of isolation and extraction. Hydroxylase activity may be detected by more sensitive means such as by tritium release [171].

Summing up, one is left with the uncomfortable conclusion that catechol oxidase remains an enzyme in search of a function. Among the possible roles might be an involvement in photosynthesis, by acting as an oxygen buffer or scavenger [1] or via effects on the Mehler reaction [13]. It cannot be overstressed that investigations in this respect must become much more rigorous and should locate both enzyme and substrate cytologically by much more sophisticated means and must assign its function at a finite and defined developmental stage. Proof of function must eventually be accompanied by studies in which mutants and absolutely specific inhibitors, such as antibodies, are used. With the increasing use of tissue suspension and callus cultures, some of these aims should now be attainable.

STRUCTURE AND REACTION MECHANISM

Laccase

The entire problem of laccase structure and reaction mechanism has been reviewed authoritatively and in great detail in a number of articles [2, 7, 172, 173] which cover the topic very comprehensively up to about 1982. Although a number of laccases have been rather fully characterized from the point of view of MW, amino acid composition, multiplicity of subunits and their carbohydrate moiety [90, 92, 95, 96, 101, 103, 174, 175], work on the reaction mechanism has been confined to one higher plant enzyme, that from *Rhus*, and two fungal ones, from *Polyporus* and *Podospora*. Much of the recent work has focussed on the three types of copper site present in the enzyme. The type 2 copper site, which is the site of substrate binding [see for example 47] has been investigated, using the technique of depletion of type 2 Cu^{2+}, which can be restored, permitting the observation of changes during interaction with substrate. However, not only the site 2 Cu can be depleted [47, 52]. Type 1 Cu has been removed and can be partly replaced by Hg^{2+}. Replacement of type 1 Cu^{2+} by Hg^{2+} resulted in greatly reduced activity [51]. The type 1 site is directly accessible to solvent including water [44, 176]. The coordination of the different types of Cu site differs, type 1 being apparently linked to cysteine, histidine and probably methionine [4, 45, 177] in *Polyporus*. The studies on type 2 Cu show it to be liganded by two to three nitrogens and one or two oxygens. Type 1 and type 2 sites interact. The type 3 and type 2 sites can be bridged for example by azide. The coupled binuclear site in native laccase seems to be very different from the binuclear site of catechol oxidase [178]. How type 3 Cu is affected by depletion of type 2 is still being debated [40, 53]. In this respect the observations that in the native enzyme, as isolated, part of the type 3 site remained reduced (about 25%) is important. This may mean that some of the results of the studies using oxidants such as H_2O_2 may be due to oxidation of these sites rather than as previously interpreted [179]. Electrons appear to enter the laccase system via site 1 and are transferred to site 2 at least as far as fungal laccase is concerned. In *Rhus* laccase, at certain pHs entry may also be via site 2. However, type 2 Cu^{2+} depletion may entirely prevent electron transfer between sites 1 and 3 [48]. Types 2 and 3 appear to be the Cu sites responsible for O_2 reduction [180]. The two sites are apparently cooperative [48], but much is still unclear in this respect.

A number of recent papers emphasize the complexity of all interpretations. Laser irradiation does not reduce reduction of copper, but induces changes which may be

due to breakage of a cysteine-type 1 Cu^{2+} bond. This irradiation also affects site 3 Cu. The native enzyme was found to be more stable to laser irradiation than type 2 Cu^{2+} depleted enzyme [49]. Enzyme–protein conformation plays a role and changes as the type 3 Cu^{2+} centre becomes reduced and these changes at site 3 lead to changes in site 1 Cu^{2+} [56]. The oxidized enzyme actually exists in two kinetically different forms, an active and an inactive one [181], which are not in equilibrium in the oxidized state [56]. Changes at site 2 do not appear to affect site 1 Cu^{2+} [44]. The close relationship between the copper sites is indicated by the fact that the binuclear Cu^{2+} site is disturbed if type 2 Cu^{2+} is removed, perhaps by increasing the Cu Cu distance in the centre. Not only is this observation important in itself, it may also make it necessary to reinterpret some of the results obtained using the copper depletion technique [50]. It is becoming clear that the type 2 and type 3 centres are not separated. The type 3 centre loses its functional integrity when type 2 Cu^{2+} is depleted [182]. The pH dependence of the reduction step above pH 8.0 has been described and indicates that they occur after oxygen binding. Protic equilibria appear to be involved, if it is assumed that the rate limiting reductant step is reduction of type 1 Cu^{2+}. Recently it has been proposed that a trinuclear Cu site exists [178] which is able to bind and bridge small molecules. Oxygen binding may involve three electron reduced dioxygen, and depends on the type 2 and type 3 sites being close together.

Clearly it is very difficult to summarize these new results on the catalytic mechanism of laccase. The reviews already mentioned present a synthesis of many findings [5, 6] which will, however, require modification in the light of new results. Differences of interpretation of known results still exist. It is not absolutely clear whether the models which apply to *Rhus* laccase can be applied without modification to the fungal laccase. An element of doubt still exists on this point [43, 55]. Certainly it must be remembered that the two types of laccase differ appreciably in their substrate specificity [1]. Subtle differences exist in the protein coupling of type 1 Cu^{2+} between lac tree and fungal laccase. Coupling is less in the tree laccase as is the reduction potential at this site [45]. There are appreciable differences in the carbohydrate content of the various laccases. The significance of this part of the molecule has not yet been considered in the analyses of reaction mechanisms. It may be that the carbohydrate is concerned with enzyme solubility or excretion, but at present evidence is lacking.

Catechol oxidase

There have been very few significant additions to the study of the structure and mechanism of the reaction of higher plant catechol oxidases in recent years. Only a few enzymes have been completely purified and characterized [25, 32, 33, 75, 77, 78]. The results have in general confirmed the previous knowledge of the structure and properties of the enzyme. The most detailed study [32] has led to the preparation of a very stable enzyme with few electrophoretic bands, indicating that most of the multiplicity, so often observed, is due to secondary reactions (tanning, etc) as also suggested by others [33].

In contrast to the rather poor progress on higher plant catechol oxidase, very significant advances have been made on fungal catechol oxidase. A series of papers by Lerch and co-workers have unravelled the complete amino acid sequence of *Neurospora* tyrosinase (catechol oxidase) [57, 58, 184–189]. A model of the site of interaction with the phenolic substrate, mono- or diphenol, has been proposed [84] based on their binuclear centre of the type 3 copper. This closely resembles the model for haemocyanin [58]. Photooxidation of the apoenzyme leads to an inability to reactivate it with Cu^{2+}. Histidine residues appear to ligand at least one of the two active site copper atoms. A thioester between cysteine and histidine residues may be concerned in the regulation of enzyme activity [185], although the ester does not actually bind Cu^{2+}. The full description of the primary structure of the enzyme from *Neurospora* [187–189] is undoubtedly a major step in understanding its mechanism of action. Inhibitors such as azide and mimosine displace peroxide from oxytyrosine and this reaction has been used as a probe for the enzyme mechanism [58]. Suggestions that superoxide might be involved in the hydroxylation reaction have been shown to be incorrect [190]. It has also been shown that copper chelators protect catechol oxidase against inactivation by peroxide [191] and apparently Cu^{2+} is necessary for enzyme inactivation by peroxide, which is not mediated by an OH radical.

The overall reaction mechanism of at least *Neurospora* catechol oxidase has been described in models by Lerch [8] and Solomon [7], which are now generally accepted. These models include a binuclear centre and copper liganded in part by histidine. Monophenols bind to one of the Cu^{2+} atoms, while diphenols bind to both of them. The reaction mechanism appears to be similar to that of haemocyanin, while that of laccase resembles that of ceruloplasmin.

Despite very significant progress, some major questions remain. The structure of only one catechol oxidase has been established. *Agaricus* catechol oxidase is composed of two dissimilar subunits as first reported by Mason [192] and this has been confirmed [193]. In contrast the *Neurospora* enzyme, whose structure is now known, is composed of similar subunits [194]. The question then arises whether the mechanism described for *Neurospora*, the copper binding and various other features also apply to the *Agaricus* enzyme. Furthermore, since the subunit structure of higher plant catechol oxidase is unresolved, it cannot yet be said with certainty whether the reaction mechanism described also applies to them.

CONCLUSIONS

Catechol oxidase continues to be the subject of extensive research. There are striking differences in the degree of sophistication in the methodology used to tackle the biochemical and biophysical aspects of research on the polyphenol oxidases compared to those used in studying its biology and function, the former being far more advanced. Considerable progress has been made over recent years at the biochemical and biophysical level. From the point of view of biology and physiology advances have been very limited; the picture remains very unclear, and cardinal questions are as yet unanswered. These problems provide a challenge to the plant biologist, both because of the intrinsic interest in many of the problems and because of the continued economic importance of the enzyme.

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