Stereoselective Bimolecular Phenoxy Radical Coupling by an Auxiliary (Dirigent) Protein Without an Active Center

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Stereoselective Bimolecular Phenoxy Radical Coupling by an Auxiliary (Dirigent) Protein Without an Active Center

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The regio- and stereospecificity of bimolecular phenoxy radical coupling reactions, of especial importance in lignin and lignan biosynthesis, are clearly controlled in some manner in vivo; yet in vitro coupling by oxidases, such as laccases, only produce racemic products. In other words, laccases, peroxidases, and comparable oxidases are unable to control regio- or stereospecificity by themselves and thus some other agent must exist. A 78-kilodalton protein has been isolated that, in the presence of an oxidase or one electron oxidant, effects stereoselective bimolecular phenoxy radical coupling in vitro. Itself lacking a catalytically active (oxidative) center, its mechanism of action is presumed to involve capture of *E*-coniferyl alcohol-derived free-radical intermediates, with consequent stereoselective coupling to give (+)-pinoresinol.

Bimolecular phenoxy radical coupling is involved in numerous biological processes, including lignin (*1*), lignan (*2, 3*), and suberin (*4*) biosynthesis in vascular plants, fruiting body development in fungi (*5*), and insect cuticle melanization and sclerotization (*6*), as well as in the formation of aphid pigments (*7*) and algal cell wall polymers (*8*).

In contrast to the marked specificity observed for these varied biological systems, all previously described chemical (*9*) and enzymatic (*10*) bimolecular phenoxy radical coupling reactions in vitro have lacked strict regio- and stereospecific control. That is, if chiral centers are introduced during coupling in vitro, the products are racemic, and different regiochemistries can result if more than one potential coupling site is present. Thus, the ability to generate a particular enantiomeric form or a specific coupling product in vitro is not under explicit control. Nonetheless, bimolecular phenoxy radical coupling in vivo can lead to well-defined biopolymers and oligomers, such as melanins, lignins, and lignans, although the mechanism has been unclear. The matter is further complicated because a large number of oxidative enzymes with broad substrate specificity that exist in nature have been attributed narrow physiological functions. For example, in lignification, some six distinct oxidases (*1, 11*), including peroxidases and laccases, have been assigned roles in lignin synthesis based on their abilities to oxidize monolignols (lignin precursors). Accordingly, one-electron oxidation of the monolignol, *E*-coniferyl alcohol, results in "random" bimolecular radical coupling to afford initially dimeric products, such as (±)-dehydrodiconiferyl alcohols, (±)-pinoresinols, and (±)-guaiacylglycerol 8-*O*-4′-coniferyl alcohol ethers (Fig. 1A). Further oxidative coupling with monolignols then gives rise to the macromolecular lignins. It is inconceivable, however, that lignin formation would be left to the vagaries of such a wide range of enzymes, or be realized in a haphazard manner.

In addition to lignins, vascular plants contain a widely distributed, structurally diverse class of dimeric phenylpropanoid products known as lignans (*2, 12*). They are considered to arise via bimolecular phenoxy radical coupling (*13*) but under conditions where both the regio- and stereochemistries are explicitly controlled in order to account for their observed optical activities. Significantly, only a relatively small number of different bimolecular coupling modes are observed, with the 8,8′-linkage being the most prevalent (*2, 12*).

To confer stereospecificity in 8,8′-linked lignan formation, we have found that a coupling agent, a 78-kD protein, is involved. This protein has no detectable catalytically active oxidative center and apparently serves only to bind and orientate the coniferyl alcohol-derived free radicals, which then undergo stereoselective coupling. The formation of free radicals, in the first instance, requires the oxidative capacity of either a nonspecific oxidase or even a nonenzymatic single-electron oxidant.

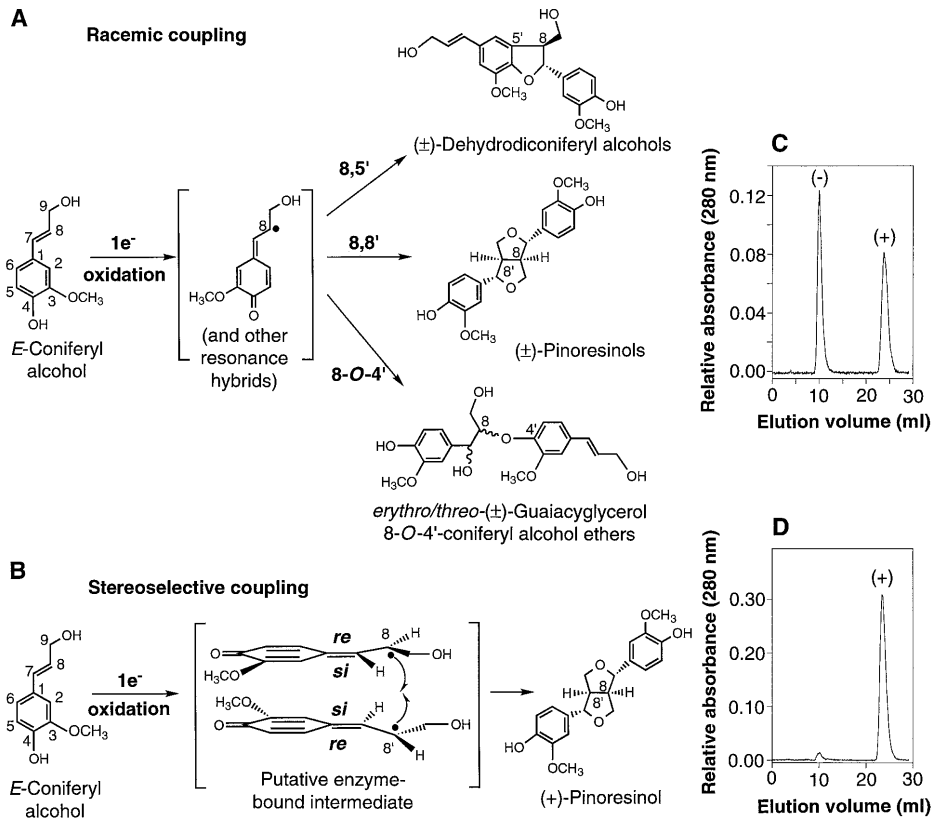

Fig. 1. Bimolecular phenoxy radical coupling products from *E*-coniferyl alcohol. (**A**) Dimeric lignans formed via "random" coupling. (**B**) Stereoselective coupling to give (+)-pinoresinol. (**C** and **D**) HPLC profiles show chirality of pinoresinol obtained for each case, respectively. [See (*16*) for elution details.]

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Initial assays in which crude "cell wall preparations" from *Forsythia suspensa* were used (*3*, *14*) revealed that entry into the various 8,8′-lignan skeleta occurs by coupling two achiral molecules of *E*-coniferyl alcohol to give (+)-pinoresinol (Fig. 1B). Both radical intermediate species, presumed bound and orientated at the supposed "(+)-pinoresinol synthase" (+PS) active site, approach each other from their *si* faces.

Significant difficulties were encountered in the solubilization of the putative +PS but these were overcome by using a potassium phosphate buffer extraction to remove readily soluble proteins from the cell-wall enriched homogenate. The remaining residual plant debris was consecutively extracted first with chilled acetone at −20°C and then potassium phosphate buffer containing 1% Triton X-100. After such treatments, the +PS activity was readily solubilized in 1 M NaCl (*15*).

Precipitation of the +PS by ammonium sulfate (40 to 80% saturation) gave a preparation that was subjected to cation exchange [MonoS and perfusion (POROS SP-M)] and gel filtration (S200) chromatography (*15*). In the initial MonoS chromatographic step, several oxidases were first eluted, all of which catalyze nonspecific oxidations of *E*-[9-^{3}H]coniferyl alcohol leading to racemic dimers. In contrast, fractions capable of engendering (+)-pinoresinol formation eluted later, when 333 mM Na_2SO_4 in 40 mM MES-NaOH buffer (pH 5.0) was used as eluent. These fractions were combined and applied to a POROS SP-M column, the elution from which with a linear gradient of Na_2SO_4 (0 to 0.7 M) gave four overlapping fractions (I to IV) as shown (Fig. 2A).

The four fractions (I to IV) from the POROS SP-M chromatographic step were individually rechromatographed (see Fig. 2, B and C, for profiles of fractions I and III, respectively), each being subsequently assayed for +PS activity with *E*-[9-^{3}H]coniferyl alcohol as substrate for 1 hour (*16*). Fraction I had very little +PS activity (< 5% of total activity loaded onto the POROS SP-M column), whereas fraction III catalyzed nonspecific oxidative coupling to give the (±)-dehydrodiconiferyl alcohols, (±)-pinoresinols, and (±)-*erythro/threo* guaiacylglycerol 8-*O*-4′-coniferyl alcohol ethers displayed in Fig. 1A. When fractions I and III were combined, however, the original +PS synthesizing activity was fully restored, that is, bimolecular coupling was reestablished with complete stereoselectivity.

Subsequent gel filtration (S200) chromatography of fraction I gave a protein of native molecular weight ~78 kD, whereas SDS–polyacrylamide gel electrophoresis showed a single band at ~27 kD (*15*), suggesting that the native protein exists as a trimer. Isoelectric focusing of the native protein on a polyacrylamide gel (pH 3 to 10 gradient) revealed the presence of six bands. After isoelectric focusing, each of these bands was electroblotted onto a polyvinylidene fluoride (PVDF) membrane and subjected to amino-terminal sequencing, which established that all had similar sequences indicating a series of isoforms. The ultraviolet-visible spectrum of the protein had only a characteristic protein absorbance at 280 nm with a barely perceptible shoulder at ~330 nm (*15*). Inductively coupled plasma (ICP) analysis gave no indication of any metal being present in the protein. Thus, the 78-kD protein lacks any detectable catalytically active (oxidative) center.

Attention was next directed to the oxidase preparation (fraction III). Although not purified to electrophoretic homogeneity, the electron paramagnetic resonance (EPR) spectrum of this protein preparation resembled that of a typical plant laccase. We then studied the fate of *E*-[9-^{3}H]coniferyl alcohol (2 μmol ml^{-1}, 14.7 kBq) in the presence of, respectively, the auxiliary oxidase (fraction III, Fig. 3A), the 78-kD protein (Fig. 3B), and both fraction III and the 78-kD protein together (Fig. 3C) (*16*). With the fraction III preparation alone, only nonspecific bimolecular radical coupling occurs to give the (±)-dehydrodiconiferyl alcohols, (±)-pinoresinols, and (±)-*erythro/threo* guaiacylglycerol 8-*O*-4′ coniferyl alcohol ethers depicted in Fig. 1A. With the 78-kD protein by itself, however, a small amount of (+)-pinoresinol formation (<5% over 10 hours) was observed, this being presumed to result from residual traces of oxidizing capacity in the preparation (see below).

When both fraction III and the 78-kD protein were combined, full catalytic activity and regio- and stereospecificity in the product was reestablished, whereby essentially only (+)-pinoresinol was formed. Note also that with fraction III alone, and when fraction III was combined with the 78-kD protein, the rates of substrate depletion and dimeric product formation were nearly identical. Moreover, essentially no turnover of the dimeric lignan products occurred in either case in the presence of the oxidase, during the time period (8 hours) examined (Fig. 3, A and C): subsequent

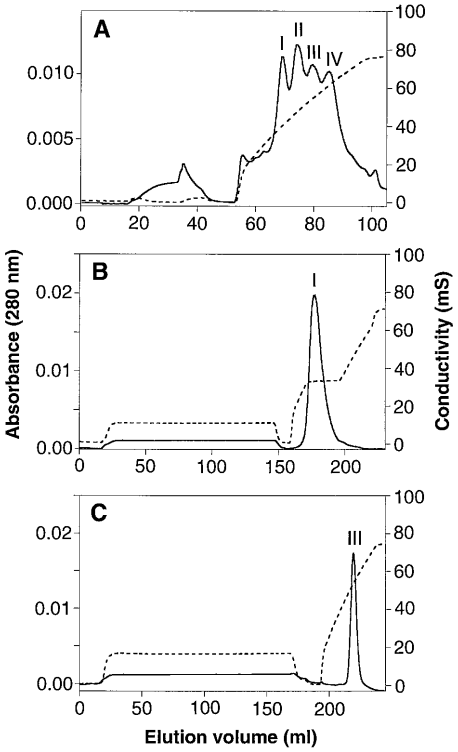


Fig. 2. Fractionation of protein mixture catalyzing (+)-pinoresinol formation by perfusion (POROS SP-M) chromatography. (**A**) Separation of proteins into four overlapping fractions I-IV, (**B**) purified fraction I, and (**C**) purified fraction III. [See (*15*) for elution details.]

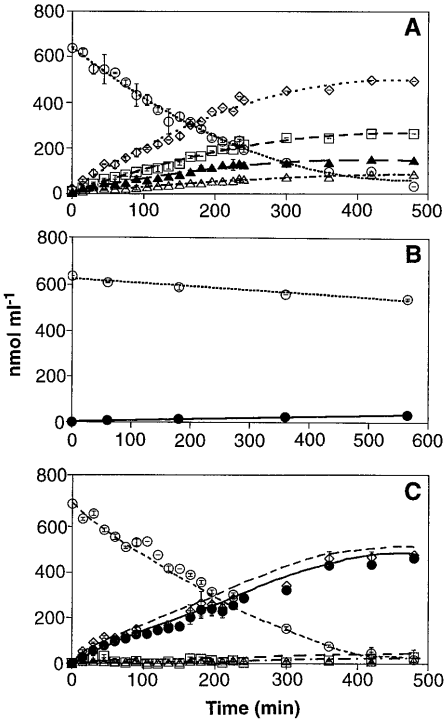


Fig. 3. Time courses for *E*-coniferyl alcohol depletion and formation of corresponding lignans during incubation in presence of: (**A**) fraction III (12 μg protein ml^{-1}); (**B**) dirigent protein (770 pmol ml^{-1}); and (**C**) fraction III (12 μg protein ml^{-1}) and dirigent protein (770 pmol ml^{-1}) together. ○, coniferyl alcohol (calculated as dimer equivalents); ●, (+)-pinoresinol; ▲, (±)-pinoresinols; □, (±)-dehydrodiconiferyl alcohols; △, (±)-*erythro/threo* guaiacylglycerol 8-*O*-4′-coniferyl alcohol ethers; ◇, total of all lignans. [Values in (C) are corrected for +PS activity noted in (B), that is, <5% over 10 hours.]

Table 1. Effect of dirigent protein on product distribution from *E*-coniferyl alcohol oxidized by ammonium peroxydisulfate (6-hour assay).

Oxidant	Dirigent protein (770 pmol ml^{-1})	*E*-Coniferyl alcohol in dimer equivalents depleted (nmol ml^{-1})	(±)-Guaiacylglycerol 8-*O*-4′-coniferyl alcohol ethers (nmol ml^{-1})	(±)-Dehydrodiconiferyl alcohols (nmol ml^{-1})	(±)-Pinoresinols (nmol ml^{-1})	(+)-Pinoresinol (nmol ml^{-1})	Total dimers (nmol ml^{-1})
Ammonium peroxydisulfate (1 μmol ml^{-1})	Absent	200 ± 4	10 ± 1	35 ± 2	16 ± 0	0	61 ± 3
	Present	250 ± 55	6 ± 0	13 ± 1	0	130 ± 10	149 ± 11
Ammonium peroxydisulfate (10 μmol ml^{-1})	Absent	860 ± 30	90 ± 4	250 ± 10	135 ± 4	0	475 ± 17
	Present	1030 ± 25	30 ± 1	90 ± 3	0	450 ± 10	570 ± 14
Dirigent protein	Present	61 ± 20	5 ± 1	8 ± 1	0	55 ± 1	68 ± 3

dimer oxidation does not occur when *E*-coniferyl alcohol, the preferred substrate, is still present in the assay mixture. The 78-kD protein therefore appears to determine the specificity of the bimolecular phenoxy radical coupling reaction. We thus propose to describe this new class of proteins as dirigent proteins (Latin: *dirigere*, to align or guide). Gel filtration studies were also carried out with mixtures of the dirigent and fraction III proteins, in order to establish whether any detectable protein-protein interaction might account for the stereoselectivity, but no evidence in support of complex formation (that is, to higher molecular size entities) was observed.

We then determined the effect that the dirigent protein would have on plant laccase-catalyzed monolignol coupling. *E*-[9-^{3}H]Coniferyl alcohol (4 μmol ml^{-1}, 29.3 kBq) was incubated with a 120-kD laccase (previously purified from *Forsythia intermedia* stem tissue) over a 24-hour period, in the presence and absence of the dirigent protein (*16*). As before, incubation with laccase alone gave only racemic dimeric products, with (±)-dehydrodiconiferyl alcohols predominating (Fig. 4A). In the presence of the dirigent protein, however, the process was now primarily stereoselective, affording (+)-pinoresinol (Fig. 4B), rather than being nonspecific as observed when only laccase was present. The rates of both *E*-coniferyl alcohol (substrate) depletion and the formation of the dimeric lignans, respectively, were similar with and without the dirigent protein (*17*). Notably, when the oxidizing capacity (that is, laccase concentration) was lowered fivefold, only (+)-pinoresinol formation was observed. Thus, complete stereoselectivity is preserved when the oxidative capacity does not exceed a point where the dirigent protein is saturated. Assays were also conducted with *E*-[9-$^2H_2$, OC^2H_3]coniferyl alcohol and the dirigent protein in the presence of laccase (*18*). After incubation, the newly formed pinoresinol was consecutively purified by reversed-phase and chiral column high-performance liquid chromatography (HPLC), with the eluent from the latter subjected to mass spectrometric analysis. Liquid chromatography–mass spectrometry (LC-MS) analysis of the resulting (+)-pinoresinol (>99% enantiomeric excess, Fig. 5B) gave a molecular ion with a mass-to-charge ratio (*m/z*) of 368 (Fig. 5A), thus establishing the presence of 10 ^2H atoms and verifying that together the laccase- and dirigent protein–catalyzed stereoselective coupling of *E*-[9-$^2H_2$, OC^2H_3]coniferyl alcohol.

Other auxiliary one-electron oxidants can also facilitate stereoselective coupling with the dirigent protein. Ammonium peroxydisulfate readily undergoes homolytic cleavage (*19*) and is routinely used as a one-electron oxidant in acrylamide polymerization. Ammonium peroxydisulfate was first incubated with *E*-[9-^{3}H]coniferyl alcohol (4 μmol ml^{-1}, 29.3 kBq) for 6 hours (*16*). Nonspecific bimolecular radical coupling was observed, to afford predominantly (±)-dehydrodiconiferyl alcohols as well as the other racemic lignans (Table 1). However, when the dirigent protein was added, the stereoselectivity of coupling was dramatically altered to give primarily (+)-pinoresinol at both concentrations of oxidant, together with small amounts of racemic lignans. This result established that even an inorganic oxidant, such as ammonium peroxydisulfate, could promote (+)-pinoresinol synthesis in the presence of the dirigent protein, even if it was not oxidatively as selective toward the monolignol as was the fraction III oxidase or laccase.

Next, the effects of incubating *E*-coniferyl alcohol (4 μmol ml^{-1}, 29.3 kBq) with flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD) were investigated because, in addition to their roles as enzyme cofactors, they can also oxidize

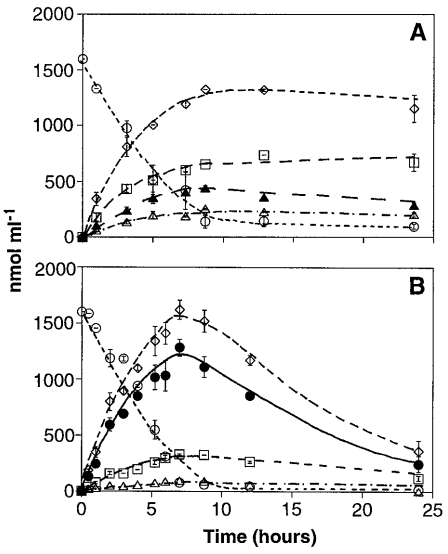


Fig. 4. Time courses for *E*-coniferyl alcohol depletion and formation of corresponding lignans during incubation in presence of (**A**) *Forsythia intermedia* laccase (10.7 pmol protein ml^{-1}) and (**B**) *F. intermedia* laccase (10.7 pmol protein ml^{-1}) and dirigent protein (770 pmol ml^{-1}) together. [See Fig. 3 for symbol legend; (B) is corrected for residual activity shown in Fig. 3B.]

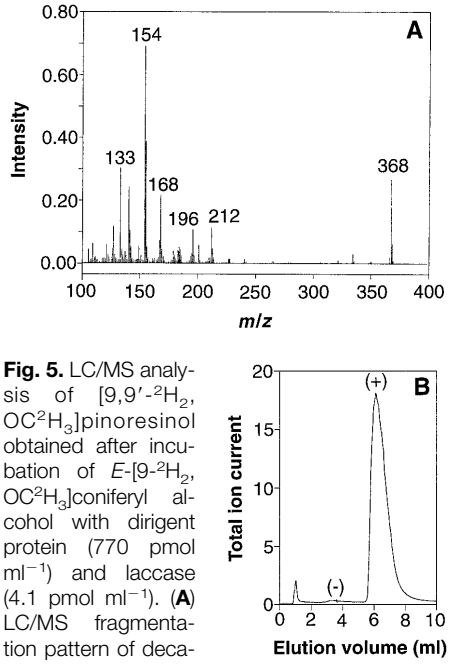


Fig. 5. LC/MS analysis of [9,9′-$^2H_2$, OC^2H_3]pinoresinol obtained after incubation of *E*-[9-$^2H_2$, OC^2H_3]coniferyl alcohol with dirigent protein (770 pmol ml^{-1}) and laccase (4.1 pmol ml^{-1}). (**A**) LC/MS fragmentation pattern of decadeuterated pinoresinol with molecular ion (*m/z*) = 368. (**B**) Total ion current showing relative ratio of (+)- and (−)-forms of pinoresinol after elution from Chiralcel OD column.

Table 2. Effect of dirigent protein on coupling of *E*-sinapyl alcohol (6-hour assay).

Oxidant	Dirigent protein (770 pmol ml^{-1})	*E*-Sinapyl alcohol in dimer equivalents depleted (nmol ml^{-1})	Racemic (±)-syringaresinols (nmol ml^{-1})
FMN (0.5 μmol ml^{-1})	Absent	570 ± 100	290 ± 40
	Present	610 ± 110	340 ± 40
Ammonium peroxydisulfate (10 μmol ml^{-1})	Absent	1400 ± 120	1020 ± 40
	Present	1520 ± 10	1060 ± 30
Dirigent protein	Present	110 ± 10	50 ± 10

Table 3. Effect of various oxidants on formal K_m and V_{max} values for the dirigent protein (770 pmol ml^{-1}) during (+)-pinoresinol formation from *E*-coniferyl alcohol.

Oxidase or oxidant	Formal K_m (mM)	V_{max} (mol s^{-1} mol^{-1} dirigent protein)
Dirigent protein	10 ± 6	0.02 ± 0.02
Fraction III*	1.6 ± 0.3	0.10 ± 0.03
Laccase†	0.100 ± 0.003	0.0600 ± 0.0002
FMN‡	0.10 ± 0.01	0.024 ± 0.001

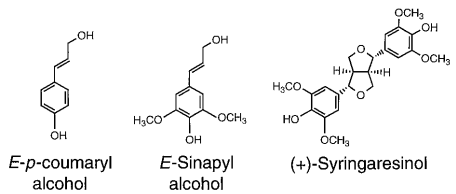
*12 μg protein ml^{-1}. †2.07 pmol ml^{-1}. ‡0.5 μmol ml^{-1}.

various organic substrates (*20*). Thus, *E*-[9-^{3}H]coniferyl alcohol was incubated with FMN and FAD, respectively, for 48 hours (*16, 21*). In every instance, *E*-coniferyl alcohol oxidation was more rapid in the presence of FMN (Fig. 6A) than FAD (Fig. 6C). Although these differences between the FMN and FAD catalyzed rates of *E*-coniferyl alcohol oxidation were not anticipated, a consistent pattern was sustained: racemic lignan products were obtained, with the (±)-dehydrodiconiferyl alcohols predominating as before. When the time courses were repeated in the presence of the dirigent protein, a dramatic change in stereoselectivity was observed (Fig. 6, B and D), where essentially only (+)-pinoresinol formation occurred. Again, the rates of *E*-coniferyl alcohol depletion, when adjusted for the traces of residual oxidizing capacity (<5% over 10 hours) in the dirigent protein preparation, were dependent only upon [FMN] and [FAD], as were the total amounts of dimers formed. When full depletion of *E*-coniferyl alcohol occurs, the corresponding lignan dimers can begin to undergo oxidative changes as a function of time; specifically, FMN is able subsequently to oxidize pinoresinol, in open solution, after the *E*-coniferyl alcohol has been fully depleted.

We found that the coupling stereoselectivity was substrate specific. Neither *E*-*p*-[9-^{3}H]coumaryl (4 μmol ml^{-1}, 44.5 kBq) or *E*-[8-^{14}C]sinapyl alcohols (4 μmol ml^{-1}, 8.3 kBq), which differ from *E*-coniferyl alcohol only by a methoxyl group substituent on the aromatic ring, yielded stereoselective products when incubated for 6 hours with FMN and ammonium peroxydisulfate, respectively, in the presence and absence of the dirigent protein (*22*). *E*-Sinapyl alcohol readily underwent coupling to afford syringaresinol, but chiral HPLC analysis revealed that the resulting products were, in every instance, racemic (Table 2). Interestingly, by itself the 78-kD dirigent protein preparation catalyzed a low level of dimer formation, as previously noted, but only gave rise to racemic (±)-syringaresinol formation, which is presumably a consequence of the residual traces of contaminating oxidizing capacity present in the protein preparation. In an analogous manner, no stereoselective coupling was observed with *E*-*p*-coumaryl alcohol as substrate. That is, only *E*-coniferyl alcohol undergoes stereoselective coupling in the presence of the dirigent protein. The low level of racemic syringaresinols obtained with the dirigent protein preparation alone confirms that traces of contaminating oxidase activity were present. Given the marked substrate specificity of the dirigent protein for *E*-coniferyl alcohol, it will be of considerable interest to determine how it differs from that affording (+)-syringaresinol in *Eucommia ulmoides* (*23*).

In regard to a mechanism for stereoselective coupling, three distinct possibilities can be envisaged. The most likely is that the oxidase or oxidant generates free-radical species from *E*-coniferyl alcohol, and that the latter are the true substrates that bind to the dirigent protein prior to coupling. The other two possibilities would require that *E*-coniferyl alcohol molecules are bound and orientated on the dirigent protein, thereby ensuring that only (+)-pinoresinol formation occurs upon subsequent oxidative coupling: this could occur either if both substrate phenolic hydroxyl groups were exposed so that they could readily be oxidized by an oxidase or oxidant, or if an electron transfer mechanism were operative between the oxidase or oxidant and an electron acceptor site or sites on the dirigent protein.

Among the three alternative mechanisms, three lines of evidence suggest "capture" of phenoxy radical intermediates by the dirigent protein. (i) The rates of both substrate depletion and product formation are largely unaffected by the presence of the dirigent protein. If capture of the free-radical intermediates is the operative mechanism, then the dirigent protein would only affect the specificity of coupling when single-electron oxidation of coniferyl alcohol is rate determining. (ii) An electron transfer mechanism is currently ruled out, because we observed no new ultraviolet-visible chromophores in either the presence or absence of an auxiliary oxidase or oxidant, under oxidizing conditions. (iii) Preliminary kinetic data (Table 3) support the concept of free-radical capture based on the formal values of Michaelis constant (K_m) and maximum velocity (V_{max}) characterizing the conversion of *E*-coniferyl alcohol into (+)-pinoresinol, with the dirigent protein alone and in the presence of the various oxidases or oxidants (*24*). If free-radical capture by the dirigent protein is the operative mech-



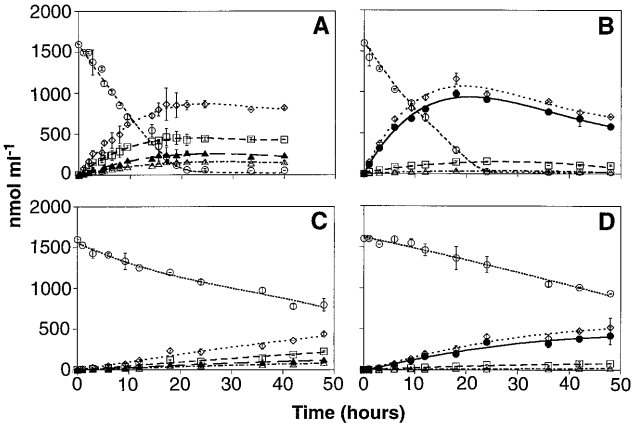


Fig. 6. Time courses for *E*-coniferyl alcohol depletion and formation of corresponding lignans during incubation in presence of (**A**) FMN (0.5 μmol ml^{-1}), (**B**) FMN (0.5 μmol ml^{-1}) and dirigent protein (770 pmol ml^{-1}) together, (**C**) FAD (0.5 μmol ml^{-1}), and (**D**) FAD (0.5 μmol ml^{-1}) and dirigent protein (770 pmol ml^{-1}) together. [See Fig. 3 for symbol legend; (B) and (D) are corrected for residual activity shown in Fig. 3B.]

anism, the Michaelis-Menten parameters obtained will only represent formal rather than true values, because the highest free-energy intermediate state during the conversion of *E*-coniferyl alcohol into (+)-pinoresinol is still unknown and the relation between the concentration of substrate and that of the corresponding intermediate free radical in open solution has not been delineated.

Bearing these qualifications in mind, we estimated formal K_m and V_{max} values for the dirigent protein preparation. As noted earlier, it was capable of engendering formation of low levels of both (+)-pinoresinol from *E*-coniferyl alcohol and racemic (±)-syringaresinols from *E*-sinapyl alcohol because of traces of contaminating oxidizing capacity. With this preparation (Table 3), a formal K_m of 10 ± 6 mM and V_{max} of 0.02 ± 0.02 mol s^{-1} mol^{-1} were obtained. However, with addition of fraction III, laccase, and FMN, the formal K_m values (mM) were reduced to 1.6 ± 0.3, 0.100 ± 0.003, and 0.10 ± 0.01, respectively (*25*), whereas the V_{max} values were far less affected at these concentrations of auxiliary oxidase or oxidant. These preliminary kinetic parameters are in harmony with the finding that dirigent protein does not substantially affect the rate of *E*-coniferyl alcohol depletion in the presence of fraction III, laccase, and FMN (Figs. 3, 4, and 6). Both sets of results are together in accord with the working hypothesis that the dirigent protein functions by capturing free-radical intermediates that then undergo stereoselective coupling.

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15. Supplementary material describing protein solubilization and purification and a figure showing a gel filtration profile and ultraviolet-visible spectra can be accessed on the World Wide Web at http://www.science-mag.org/science/feature/beyond/#XXXX and can be ordered from AAAS (see any current masthead for ordering information).
16. General *E*-coniferyl alcohol coupling assays. Each assay consisted of *E*-[9-^{3}H]coniferyl alcohol (4 μmol ml^{-1}, 29.3 kBq, 7.3 MBq mol $liter^{-1}$; or 2 μmol ml^{-1}, 14.7 kBq with fraction III), the 78-kD dirigent protein, an oxidase or oxidant, or both [final concentrations: 770 pmol ml^{-1} dirigent protein; 10.7 pmol protein ml^{-1} *Forsythia* laccase; 12 μg protein ml^{-1} fraction III; 0.5 μmol ml^{-1} FMN; 0.5 μmol ml^{-1} FAD; 1 and 10 μmol ml^{-1} ammonium peroxydisulfate] in buffer (0.1 M MES-HEPES-sodium acetate, pH 5.0) to a total volume of 250 μl. The enzymatic reaction was initiated by addition of *E*-[9-^{3}H]coniferyl alcohol. Controls were performed in the presence of buffer alone. After 1 hour incubation at 30°C while shaking, the assay mixture was extracted with ethyl acetate (EtOAc, 500 μl) containing (±)-pinoresinols (7.5 μg), (±)-dehydrodiconiferyl alcohols (3.5 μg), and *erythro/threo* (±)-guaiacylglycerol 8-*O*-4′-coniferyl alcohol ethers (7.5 μg) as radiochemical carriers and ferulic acid (15.0 μg) as an internal standard. After centrifugation (13,800*g*, 5 min), the EtOAc-soluble components were removed and the extraction procedure repeated with EtOAc (500 μl). The EtOAc-soluble components from each assay were combined, the solutions evaporated to dryness in vacuo and redissolved in methanol-water solution (1:1; 100 μl) with an aliquot (50 μl) thereof subjected to reversed-phase column chromatography (Waters, Nova-Pak C_{18}, 150 mm by 3.8 mm). The elution conditions were as follows: acetonitrile/3% acetic acid in H_2O (5:95) from 0 to 5 min, then linear gradients to ratios of 10:90 between 5 and 20 min, then to 20:80 between 20 and 45 min, and finally to 50:50 between 45 and 60 min, at a flow rate of 8.8 ml min^{-1} cm^{-2}. Fractions corresponding to *E*-coniferyl alcohol, *erythro/threo* (±)-guaiacylglycerol 8-*O*-4′-coniferyl alcohol ethers, (±)-dehydrodiconiferyl alcohols, and (±)-pinoresinols were individually collected, aliquots removed for liquid scintillation counting, and the remainder freeze-dried. Pinoresinol-containing fractions were redissolved in methanol (100 μl) and subjected to chiral column chromatography (Daicel, Chiralcel OD, 50 mm by 4.6 mm) with a solution of hexanes and ethanol (1:1) as the mobile phase (flow rate 3 ml min^{-1} cm^{-2}), whereas dehydrodiconiferyl alcohol fractions were subjected to Chiralcel OF (250 mm by 4.6 mm) column chromatography eluted with a solution of hexanes and isopropanol (9:1) as the mobile phase (flow rate 2.4 ml min^{-1} cm^{-2}), the radioactivity of the eluent being measured with a flow-through detector (Radiomatic, Model A120).
17. A substantial difference was noted in the subsequent turnover of the lignan products observed after *E*-coniferyl alcohol depletion. With the laccase alone no turnover occurred, but when both proteins were present the disappearance of the products was significant. In order to understand the difference, assays were conducted where bovine serum albumin (BSA) and ovalbumin were individually added to the laccase-containing solutions at concentrations matching the weight concentrations of the dirigent protein. In this way, it was established that the differences in product turnover were simply due to stabilization of laccase activity at the higher protein concentrations, although interestingly the dirigent protein, BSA, and ovalbumin afforded somewhat different degrees of protection. The findings were quite comparable when a fungal laccase (from *Trametes versicolor*) was used in place of the plant laccase.
18. Incubation with *E*-[9-$^2H_2$, OC^2H_3]coniferyl alcohol. *E*-[9-$^2H_2$, OC^2H_3]Coniferyl alcohol (2 μmol ml^{-1}) was incubated in the presence of dirigent protein (770 pmol ml^{-1}), the purified plant laccase (4.1 pmol ml^{-1}), and buffer (0.1 M MES-Hepes-sodium acetate, pH 5.0) in a total volume of 250 μl. After 1 hour incubation, the reaction mixture was extracted with EtOAc as described in (16), but with the addition of an internal standard and omission of radiochemical carriers. After reversed-phase column chromatography, the enzymatically formed pinoresinol was collected, freeze-dried, redissolved in methanol (100 μl), and subjected to chiral column chromatography (Daicel, Chiralcel OD, 50 mm by 4.6 mm) with detection at 280 nm and analysis by mass spectral fragmentation in the EI mode (Waters, Integrity System)
19. A. Usaitis and R. Makuska, *Polymer* **35**, 4896 (1994).
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21. Enzymatic synthesis of FMN. Snake (*Naja naja atra*, Formosan cobra) venom was added to a solution of FAD (5 μmol ml^{-1} in H_2O) and, after 30-min incubation at 30°C, the enzymatically formed FMN was separated from the protein mixture by filtration through a Centricon 10 (Amicon) microconcentrator.
22. Substrate specificity. Incubations were carried out as described in (*16*) with the following modifications: *E*-*p*-[9-^{3}H]Coumaryl (4 μmol ml^{-1}, 44.5 kBq) or *E*-[8-^{14}C]sinapyl alcohols (4 μmol ml^{-1}, 8.3 kBq) were used as substrates and, after 6-hour incubation at 30°C, the reaction mixture was extracted with EtOAc but without addition of radiochemical carriers.
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24. Kinetic parameters. Assays were carried out as described in (*16*) by incubating a series of *E*-[9-^{3}H]coniferyl alcohol concentrations (between 8.00 and 0.13 μmol ml^{-1}, 7.3 MBq mole $liter^{-1}$) with dirigent protein (770 pmol ml^{-1}), alone and in presence of *Forsythia* laccase (2.1 pmol ml^{-1}), fraction III (12 μg protein ml^{-1}), or FMN (0.5 μmol ml^{-1}). Assays with dirigent protein, with or without FMN, were incubated at 30°C for 1 hour, whereas assays with *Forsythia* laccase or fraction III with or without dirigent protein were incubated at 30°C for 15 min.
25. Formal K_m and V_{max} values were calculated for the laccase and fraction III oxidase with respect to *E*-coniferyl alcohol conversion into the three racemic lignans. However, no direct comparisons can be made to the 78-kD protein because the formal K_m values involve only the corresponding oxidases. For completeness, the K_m (mM) and V_{max} (mol s^{-1} mol^{-1} enzyme) were as follows: with respect to the laccase, 0.200 ± 0.001 and 3.9 ± 0.2 for (±)-*erythro/threo* guaiacylglycerol 8-*O*-4′-coniferyl alcohol ethers, 0.3000 ± 0.0003 and 13.1 ± 0.6 for (±)-dehydrodiconiferyl alcohols, and 0.300 ± 0.0002 and 7.54 ± 0.50 for (±)-pinoresinols; with respect to the fraction III oxidase (estimated to have a native molecular weight of 80 kD), 2.2 ± 0.3 and 0.20 ± 0.03 for (+)-*erythro/threo* guaiacylglycerol 8-*O*-4-coniferyl alcohol ethers, 2.2 ± 0.2 and 0.7 ± 0.1 for (±)-dehydrodiconiferyl alcohols, and 3.7 ± 0.7 and 0.6 ± 0.1 for (±)-pinoresinols.

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