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Probing the Mysteries of Lignin Biosynthesis: The Crystal Structure of Caffeic Acid/5-Hydroxyferulic Acid 3/5-O-Methyltransferase Provides New Insights

Caffeic acid/5-hydroxyferulic acid 3/5-O-methyltransferase (COMT) is a small-molecule *S*-adenosyl-L-Met-dependent *O*-methyltransferase (OMT) that is one of the principal enzymes in the complex network of reactions that take place as part of lignin biosynthesis (Figure 1). Plants contain a wide variety of *S*-adenosyl-L-Met-dependent OMTs that act on Phe-derived substrates during the production of numerous plant "secondary" compounds in addition to lignin, such as anthocyanin flower pigments, isoflavonoid antimicrobial compounds, phytoestrogens and allelochemicals, coumarin defense compounds, and chalcone nodulation factors. Some OMTs exhibit a high degree of substrate specificity. For example, chalcone OMT (ChOMT) from alfalfa methylates the 2'-hydroxyl of 4,2',4'-trihydroxychalcone to produce 4,4'-dihydroxy-2'-methoxychalcone, which is an inducer of *Rhizobia* nodulation genes, and isoflavone OMT (IOMT) converts a putative isoflavone substrate to formononetin, a potent phytoestrogen that also is a precursor of medicarpin, the principal antifungal phytoalexin of alfalfa.

In contrast to ChOMT and IOMT, COMT exhibits broader substrate specificity. It is a bifunctional enzyme that methylates substrates at the 5-hydroxy and 3-hydroxy positions on the aromatic ring and acts (with varying affinity) on free acid, aldehyde, and alcohol 3-hydroxy and 3,5-dihydroxy phenylpropanoid substrates.

The broad specificity of COMT is one of the main reasons that monolignol biosynthesis is viewed as a highly complex network or grid, and definitive *in vivo* pathways have yet to be determined. In this issue of *The Plant Cell*, **Zubieta et al. (pages 1265–1277)** present an analysis of the crystal structure of COMT from alfalfa that



Figure 1. COMT Is One of the Key Enzymes Involved in Monolignol Biosynthesis in Angiosperms.

Analysis of the crystal structure of alfalfa COMT provides an explanation for substrate specificity and serves as a template for engineering new specificities designed to alter lignin concentration and composition in plants. (Figure courtesy of Joseph Noel.)

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helps to explain the broad specificity of this enzyme in contrast to other OMTs as well as the demonstrated preference for aldehydes and alcohols over free acid substrates and for 5-hydroxy-substituted molecules over those substituted on only the 3-position of the aromatic ring.

METHYLATION, THE SYRINGYL: GUAIACYL RATIO, AND THE LIGNIN BIOSYNTHESIS METABOLIC GRID

In dicotyledonous angiosperms, lignin is composed of guaiacyl (G) and syringyl (S) monolignol units, which differ in the degree of methylation of the phenylpropane units. G monolignol units are derived from caffeic acid (and/or its related aldehydes and alcohols) and are methylated on the 3-hydroxy position of the aromatic ring, whereas S units are derived from sinapic acid (and/or its related aldehydes and alcohols) and are methylated on both the 3- and 5-hydroxy positions. The monolignols are relatively unstable toxic compounds that do not accumulate in plant cells, but they are quickly glycosylated to produce monolignol glucosides, which are the likely storage and transport forms of the monolignol units. The glycosidic bond then is cleaved, most likely at the site of lignification, and free monolignols are polymerized into lignin via a free radical mechanism catalyzed by cell wall-bound oxidases (for review, see Whetten and Sederoff, 1995; Whetten et al., 1998).

Manipulation of the S:G ratio has been a major objective of lignin biosynthesis researchers—in particular in the pulp and paper industry—for decades. The S:G ratio is important in pulping because a higher G content requires the use of more expensive and environmentally hazardous chemicals. S units in lignin typically are linked via relatively labile ether bonds, which are chemically degraded more easily than linkages between G units. G monolignols form more highly condensed lignin with a preponderance of more stable

(and recalcitrant) biphenyl and other carbon-carbon linkages. Gymnosperms lack S monolignol units and therefore are far less desirable than the “hardwood” angiosperms for pulp and paper production.

There also is interest in manipulating lignin concentration and composition in forage grasses, such as alfalfa, because these parameters—lignin concentration in particular—have an effect on digestibility in ruminant animals (Sewalt et al., 1997). There is a high degree of variability in the S:G ratio between species and within single species, and even within individual plants, as a result of genetic, developmental, and environmental parameters. Even small reductions in the S:G ratio and in overall lignin content could lead to significant improvements in the pulping process. Thus, genetic engineering of the S:G ratio would appear to be a feasible and worthwhile goal.

The degree of methylation of the monolignol unit determines the S:G ratio. The classic historical view of lignin biosynthesis is for methylation via COMT to occur at the level of free acids. COMT is capable of the methylation of caffeic acid (hydroxylated at the 3- and 4-positions on the aromatic ring) at the 3-hydroxy position to yield ferulic acid and of 5-hydroxyferulic acid (methylated at the 3-position and hydroxylated at the 4- and 5-positions) at the 5-hydroxy position to yield sinapic acid. Ferulic acid and sinapic acid then could be converted successively to their corresponding thioesters, aldehydes and alcohols, yielding the guaiacyl and syringyl monolignols, respectively.

In recent years, there has been a growing consensus that alternative pathways for monolignol biosynthesis exist, and may even predominate in many plants, based on the discoveries that COMT can methylate substrates at the levels of aldehydes and alcohols and that another OMT, caffeoyl CoA OMT (CCoAOMT) can methylate the CoA esters of 3-hydroxy and 5-hydroxy acids (for review, see Dixon et al., 2001; Fukushima, 2001). Thus, the textbook version of lignin biosynthesis today typically is presented as a complex “metabolic grid,”

with conversion of the aromatic ring (e.g., methylation) occurring at any or all of these levels (Figure 2).

INDEPENDENT PATHWAYS REVISITED

More recently, evidence related to COMT and CCoAOMT activity has supported the notion that there may be separate (but overlapping) linear pathways to G and S monolignol units (Dixon et al., 2001; Humphreys and Chapple, 2002). In other words, although they exhibit broad substrate specificity *in vitro*, the *in vivo* activity of these enzymes may occur predominantly at one level—most likely aldehyde or alcohol instead of free acid—and against a minimal number of principal substrates (Figure 2).

COMT was isolated and characterized based on its ability to catalyze the methylation of caffeic acid (at the 3-hydroxy position) to produce ferulic acid and the methylation of 5-hydroxyferulic acid (at the 5-hydroxy position) to produce sinapic acid. In older models of lignin biosynthesis, these are presented as the major reactions catalyzed by COMT. However, it has been found that COMT from various species has a marked preference for aldehyde and alcohol substrates over free acids and for the 5-hydroxy over the 3-hydroxy position on the aromatic ring. For example, Zubieta et al. (2002) measured the K_m of alfalfa COMT for caffeic acid, 5-hydroxyferulic acid, and 5-hydroxyconiferaldehyde at 43, 10, and 5 μM , respectively. Similar K_m values for these substrates were measured previously for COMT from alfalfa (Parvathi et al., 2001) and aspen (Li et al., 2000).

Antisense inhibition of COMT to levels less than ~25% of wild-type levels has been associated with strong reductions in the S:G ratio in a number of plant species (e.g., tobacco [Atanassova et al., 1995], alfalfa [Guo et al., 2001], poplar [Lapierre et al., 1999], and aspen [Tsai et al., 1998]). The substrate preference and antisense inhibition data suggest that COMT may have a primary role in 5-hydroxy side-chain reduc-

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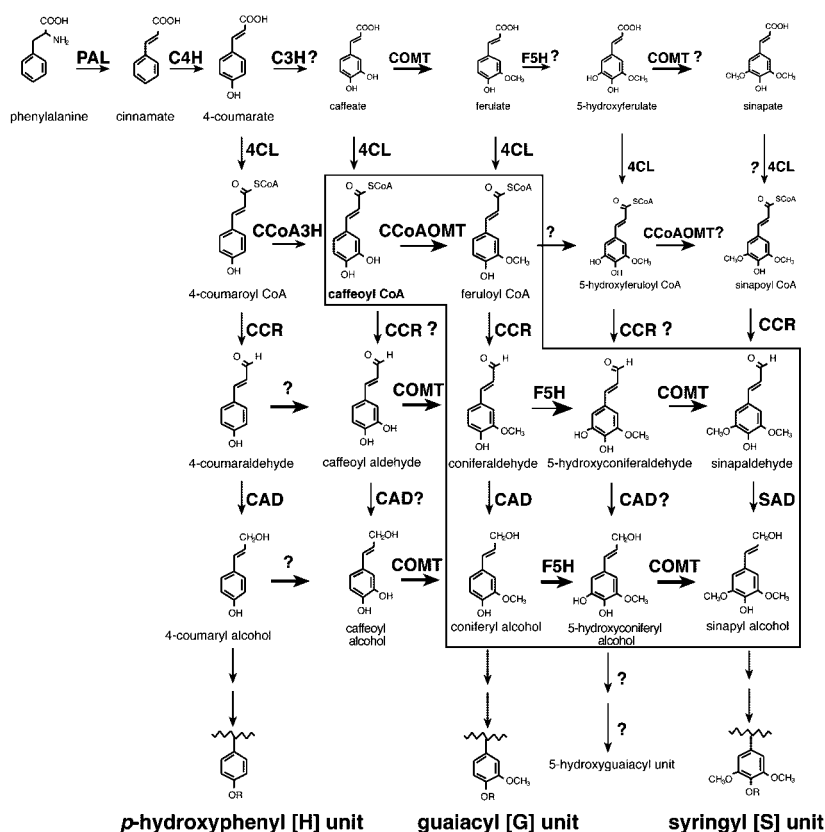


Figure 2. Metabolic Grid of Potential Monolignol Biosynthetic Reactions.

Early models of lignin biosynthesis show methylation reactions occurring at the level of free acids. The discoveries of COMT activity against aldehyde and alcohol substrates and CCoAOMT activity against CoA esters led to the development of the metabolic grid model of monolignol biosynthesis. Rather than the operation of the complete metabolic grid shown here, the most recent models (Dixon et al., 2001; Humphreys and Chapple, 2002) suggest linear, or nearly linear, pathways of methylation operating at the higher levels of side-chain modification with little or no flux through caffeic acid (e.g., boxed reactions). CAD, cinnamyl alcohol dehydrogenase; CCoA3H, caffeoyl-CoA 3-hydroxylase; CCoAOMT, caffeoyl-CoA O-methyltransferase; CCR, cinnamoyl-CoA reductase; C3H, coumarate 3-hydroxylase; C4H, cinnamate 4-hydroxylase; 4CL, 4-coumarate:CoA ligase; COMT, caffeic acid O-methyltransferase; F5H, ferulate 5-hydroxylase; PAL, Phe ammonia-lyase; SAD, sinapyl alcohol dehydrogenase. (Figure courtesy of Richard Dixon.)

tion at a later step in S unit biosynthesis (e.g., methylation of 5-hydroxyconiferaldehyde to produce sinapaldehyde), as opposed to earlier steps that could lead to both G and S biosynthesis (e.g., methylation of caffeic acid to produce ferulic acid) (Dixon et al., 2001; Humphreys and Chapple, 2002). Radiolabeling studies have shown that S lignin units can be formed in vivo from cin-

namyl alcohols (Matsui et al., 2000). It also has been observed that the COMT-mediated methylation of caffeic acid is inhibited competitively by 5-hydroxyconiferyl aldehyde in vitro, further suggesting that caffeic acid may not be a regular in vivo substrate of the enzyme (Li et al., 2000).

Interestingly, CCoAOMT activity appears to be associated mainly with the biosynthe-

sis of G monolignol. For example, Zhong et al. (1998) created transgenic tobacco with substantial reductions (caused by antisense inhibition) in COMT and/or CCoAOMT and found that a reduction in COMT was associated with a markedly reduced S:G ratio, whereas a reduction in CCoAOMT was associated with an increase in the S:G ratio. Inhibition of COMT reduced the S:G ratio without significantly altering the overall lignin concentration, whereas inhibition of CCoAOMT caused a dramatic decrease in total lignin in addition to an increase in the S:G ratio, suggesting that CCoAOMT may play a secondary role in S biosynthesis (Zhong et al., 1998).

THE STRUCTURE OF COMT OFFERS AN EXPLANATION FOR SUBSTRATE PREFERENCES

The structural analysis of alfalfa COMT by Zubieta et al. (2002) provides an explanation for the preference of the enzyme for alcohols and aldehydes and for 5-hydroxy-substituted substrates. Amino acid residues were identified that form hydrogen bonds with the 5-hydroxyl group substrate molecules, thus sequestering the substrate in close proximity to the active site, and a propanoid tail binding region of the active site was found to be related to the preference for aldehydes and alcohols over acid substrates. This analysis offers support for the notion that the principal in vivo substrate(s) of the enzyme is an aldehyde and/or alcohol, such as caffeoyl aldehyde, 5-hydroxyconiferaldehyde, and/or caffeoyl alcohol, as suggested by Parvathi et al. (2001) and Li et al. (2000). The structure of alfalfa COMT also showed a larger substrate binding pocket than did the previously analyzed structures of ChOMT and IOMT (Zubieta et al., 2001), which may explain the broader overall substrate specificity of COMT compared with other OMTs.

Based on the structural analysis of COMT, Zubieta et al. performed site-directed mutagenesis of specific amino acid residues, resulting in the production of mutant

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enzymes with altered substrate specificities. Various single amino acid substitutions produced enzymes with increased affinity for caffeic acid, a complete loss of caffeic acid binding, increased selectivity for 5-hydroxyconiferaldehyde and 5-hydroxyconiferyl alcohol, or a loss of discrimination between known substrates. It will be of great interest to test the effect of these mutant enzymes on lignin biosynthesis *in vivo* in transgenic plants.

It is important to note that COMT and CCoAOMT are not encoded by single genes; rather, they form multigene families in many plant species. For example, tobacco contains two classes of COMT and three classes of CCoAOMT, based on cDNA sequence analysis (Pinçon et al., 2001). Expression analyses suggest that tobacco COMT I is associated primarily with lignin biosynthesis, whereas COMT II may be involved in phenylpropanoid metabolism associated with plant defense responses, because it is barely detected in healthy tissue but is induced strongly during the hypersensitive response (Pellegrini et al., 1993).

Interestingly, a second form of COMT, COMT II, also has been identified in alfalfa (Inoue et al., 2000). Unlike the major form of COMT, which increases in stem internodes as the plants mature (in parallel with lignin biosynthesis) and has low affinity for caffeic acid substrate, alfalfa COMT II was found predominantly in young stem internodes and exhibited a relatively high affinity for caffeic acid. The function of COMT II is unknown, but presumably it plays a different role than does COMT I. Structural analysis of alfalfa COMT II would present an interesting contrast to that of COMT I, providing further tests of the predicted roles of specific amino acid residues in substrate recognition.

MULTIENZYME COMPLEXES IN PHENYLPROPANOID AND FLAVONOID BIOSYNTHESIS?

One mechanism that could account for single linear pathways for G and S biosyn-

thesis in the face of broad substrate specificity of COMT and other lignin biosynthetic enzymes is metabolic channeling via multi-enzyme complexes. The organization of enzymes that catalyze successive reactions in a metabolic pathway into large multienzyme complexes appears to be a common feature of cellular metabolism in many organisms, providing a mechanism for the rapid and efficient regulation of complex, multistep pathways (Winkel-Shirley, 1999). It was proposed as early as 1974 that the flavonoid and lignin biosynthetic pathways could be organized as enzyme complexes (Stafford, 1974). Using yeast two-hybrid assays, affinity chromatography, and immunoprecipitation assays, Burbulis and Winkel-Shirley (1999) demonstrated the potential for specific protein-protein interactions among the key flavonoid biosynthesis enzymes chalcone synthase, chalcone isomerase, dihydroflavonol 4-reductase, and flavonol 3-hydroxylase.

A number of other studies have suggested channeling of intermediates in phenylpropanoid and flavonoid metabolism (for review, see Winkel-Shirley, 1999; Dixon et al., 2001). Dixon et al. (2001) proposed a metabolic channel model for independent pathways to G and S monolignols in which COMT participates in a complex yielding sinapyl alcohol (the S monolignol precursor) and CCoAOMT forms part of a separate complex producing coniferyl alcohol, the precursor of G monolignol. This model is worthy of more rigorous investigation. Continued elucidation of the crystal structures of COMT, as well as CCoAOMT and the other monolignol biosynthetic enzymes, may provide a structural basis for this concept, for example, by facilitating the prediction of protein surfaces and domains that could provide for interactions among putative multienzyme complex members.

Structural analysis, such as that presented in this issue for COMT by Zubieta et al. (2002), is a critical component of efforts toward the complete understanding of pathways of metabolism, signal transduction, and the regulation of gene

expression. Structural biology is an important tool that may help us understand the functions of numerous large families of enzymes, such as the OMTs, that exist within individual species. The detailed structural characterization of proteins provides a means of understanding substrate preferences and protein-protein interactions and provides a solid basis for engineering plant metabolism toward directed goals.

Nancy A. Eckardt
News and Reviews Editor
neckardt@aspb.org

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