

## Update on Metabolism

# The Shikimate Pathway as an Entry to Aromatic Secondary Metabolism<sup>1</sup>

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The shikimate pathway is often referred to as the common aromatic biosynthetic pathway, even though nature does not synthesize all aromatic compounds by this route. This metabolic sequence converts the primary metabolites PEP and erythrose-4-P to chorismate, the last common precursor for the three aromatic amino acids Phe, Tyr, and Trp and for *p*-amino and *p*-hydroxy benzoate (Fig. 1). The shikimate pathway is found in bacteria, fungi, and plants. In monogastric animals, Phe and Trp are essential amino acids that have to come with the diet and Tyr is directly derived from Phe. Since bacteria use in excess of 90% of their metabolic energy for protein biosynthesis, for most prokaryotes, the three aromatic amino acids represent nearly the entire output of aromatic biosynthesis, and regulatory mechanisms for shikimate pathway activity are triggered by the intracellular concentrations of Phe, Tyr, and Trp. This is not so in higher plants, in which the aromatic amino acids are the precursors for a large variety of secondary metabolites with aromatic ring structures that often make up a substantial amount of the total dry weight of a plant. Among the many aromatic secondary metabolites are flavonoids, many phytoalexins, indole acetate, alkaloids such as morphine, UV light protectants, and, most important, lignin.

### THE PATHWAY

Although the enzyme-catalyzed reactions of the shikimate pathway seem to be identical for prokaryotes and eukaryotes, plants have branches off of the main pathway that so far have not been demonstrated in bacteria or fungi. In this update I will focus on the main pathway. First, I will briefly review the basic regulatory features of the shikimate pathway in bacteria and then describe what is known about these features in higher plants. Here the differences between prokaryotes and eukaryotes are rather striking. I will close with some projections into future research in this exciting field.

The main trunk of the shikimate pathway consists of reactions catalyzed by seven enzymes. The best studied of

these are the penultimate enzyme, the 5-*enol*-pyruvoyl shikimate-3-P synthase, the primary target site for the herbicide glyphosate, and the first enzyme, DAHP synthase, the enzyme that controls carbon flow into the shikimate pathway. DAHP synthase catalyzes the condensation of PEP and erythrose-4-P to yield DAHP and Pi. Even though the enzyme was discovered in *Escherichia coli* more than three decades ago and has been purified to electrophoretic homogeneity from a number of sources, the fine structure of DAHP, the product of the enzyme-catalyzed reaction, was not described until many years later as the structure given in Figure 2 (Garner and Herrmann, 1984).

### DAHP SYNTHASE OF BACTERIA

The most intensively investigated DAHP synthase has been the *E. coli* enzyme. This organism encodes three DAHP synthase isoenzymes, a Phe-sensitive, a Tyr-sensitive, and a Trp-sensitive activity. The three enzymes have been purified to homogeneity, their structural genes have been characterized, and their complete primary structures have been obtained through a combination of protein and DNA sequencing efforts. The *E. coli* DAHP synthase is a metalloenzyme. The nature of the metal ion can vary and may depend on the conditions under which the bacteria were grown. The preferred metal co-factors seem to be Fe<sup>2+</sup> and perhaps Zn<sup>2+</sup>. The metal ion plays a role in catalysis and may also affect enzyme conformation. By site-directed mutagenesis, Cys<sup>61</sup> of the *E. coli* Phe-sensitive isoenzyme, which lies in a Cys-X-X-His motif near the amino terminus of the polypeptide, has been identified as a metal ion ligand (Stephens and Bauerle, 1992).

The bacterial enzyme is regulated at the protein level by feedback inhibition and at the transcriptional level by repression. The Phe- and Tyr-sensitive isoenzymes are 50% inhibited by 13 μM Phe and 20 μM Tyr, respectively, and full inhibition is reached with about 0.1 mM amino acids. However, the inhibition of the Trp-sensitive isoenzyme by Trp never exceeds 40%, presumably to ensure in the presence of excess aromatic amino acids a sufficient supply of chorismate for synthesis of *p*-amino and *p*-hydroxy ben-

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Abbreviations: DAHP, 3-deoxy-D-arabino-heptulosonate 7-phosphate.

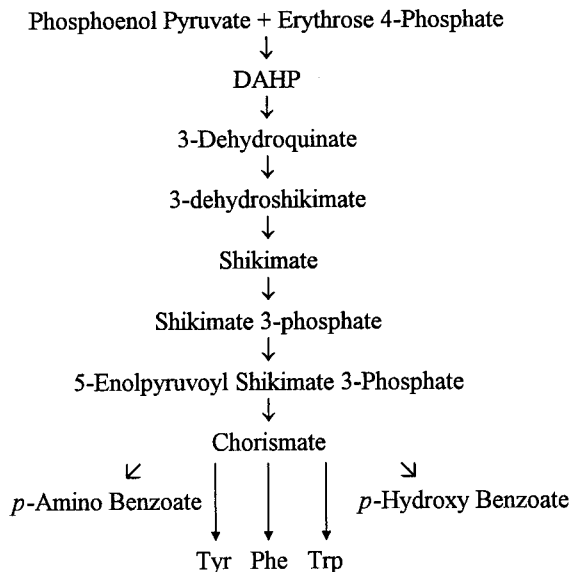


Figure 1. The shikimate pathway.

zoate, precursors of folate and enterochelin, respectively. Under most growth conditions, the intracellular concentrations of the aromatic amino acids are sufficient to render the Phe- and the Tyr-sensitive isoenzymes largely inactive. Feedback inhibition of the three individual isoenzymes is absolutely specific for the cognate amino acid, e.g. the Phe-sensitive protein is not affected by Tyr, Trp, or a combination of the two. For two of the isoenzymes, mutational analysis has identified essential amino acid residues in the feedback inhibitor-binding site (Ray et al., 1988; Weaver and Herrmann, 1990). These residues are in close proximity to a Gly-Ala-Arg-Thr sequence that constitutes part of the active site (Ray et al., 1988).

The three *E. coli* genes encoding DAHP synthases are subject to repression by the *tyr*- and *trp*-repressors complexed to the relevant aromatic amino acids. *cis*-acting regulatory mutants with lesions in the *trp*-repressor target site of the gene encoding the Trp-sensitive isoenzyme (Zurawski et al., 1981; Klig et al., 1988) and in the *tyr*-repressor target site of the gene encoding the Tyr-sensitive isoenzyme (Garner and Herrmann, 1985) defined two regulons subject to control by the two repressors. A functional connection between the *tyr*- and *trp*-regulons was inferred by the demonstration that the gene encoding the Trp-sensitive isoenzyme is controlled by both the *trp*- and the *tyr*-repressor (Muday et al., 1991). Although the transcriptional control for the expression of the three genes encoding *E. coli* DAHP synthases seems complex, feedback inhibition of the three isoenzymes is quantitatively the major regulatory mechanism controlling chorismate biosynthesis *in vivo*, as demonstrated by noninvasive NMR spectroscopy on whole living cells (Ogino et al., 1982).

#### THE STRUCTURE OF THE DAHP SYNTHASE OF HIGHER PLANTS

DAHP synthases have been partially purified from several plant species. Electrophoretically homogeneous en-

zymes have been obtained from carrot and potato. Jensen and his co-workers distinguished two forms of the enzyme in *Vigna radiata* (Rubin et al., 1982), an  $Mn^{2+}$ -stimulated and a  $Co^{2+}$ -dependent activity that have since been found in a number of other plants. Using subcellular fractionation by discontinuous Suc gradient centrifugation, the  $Mn^{2+}$  form of the enzyme was localized to the chloroplast, whereas the  $Co^{2+}$ -dependent activity was cytosolic (Ganson et al., 1986).

The  $Co^{2+}$ -dependent activity has a  $K_m$  for erythrose-4-P that is 1 order of magnitude higher than that of the  $Mn^{2+}$ -stimulated enzyme. Also, the  $Co^{2+}$ -dependent activity readily accepts other aldehydes as substrates (Doong et al., 1992). Since glycolaldehyde is a better substrate than erythrose-4-P, this enzyme might more properly be called 4,5-dihydroxy-2-oxovalerate synthase. Its involvement in DAHP synthesis should be viewed with caution, since it has been suggested to be an "enzyme of  $\alpha$ -ketoglutarate biosynthesis" (Doong et al., 1992).

The only DAHP synthases obtained in pure form from higher plants, namely the enzymes from carrot and potato, are both activated by  $Mn^{2+}$ . These enzymes appear to have absolute substrate specificity for PEP and erythrose-4-P. Therefore, the  $Mn^{2+}$ -stimulated activity may be the only one of physiological significance for DAHP synthesis.

The  $Mn^{2+}$ -stimulated DAHP synthases are oligomers like the bacterial enzymes. However, the plant enzymes are of higher mol wts; their subunits are approximately 100 amino acid residues larger. Rabbit antibodies raised against the pure potato enzyme were used to screen a cDNA library from potato cells grown in suspension culture to obtain the first plant DNA encoding this protein (Dyer et al., 1989). The resulting cDNA, called *shkA*, yielded the first primary structure of a plant DAHP synthase (Dyer et al., 1990) and has since been used as a probe to clone a second potato cDNA, called *shkB* (Zhao and Herrmann, 1992), and other complementary and genomic DNA encoding this activity from tobacco (Wang et al., 1991), *Arabidopsis thaliana* (Keith et al., 1991), and tomato (Görlach et al., 1993).

A comparison of the deduced amino acid sequences of these enzymes among themselves and with their prokaryotic homologs reveals some interesting findings. Each plant that has been analyzed in some detail contains at least two  $Mn^{2+}$ -stimulated DAHP synthase isoenzymes. These homologs of the *shkA* or the *shkB* products from different species are more similar to each other than any *shkA/shkB*

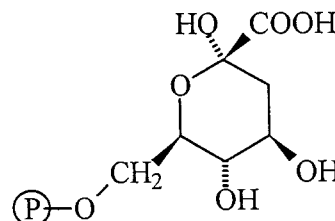


Figure 2. The structure of DAHP.

product pair of a single species. Thus, it is possible to distinguish two families of DAHP synthases that presumably arose from an early gene duplication event in a common ancestor of *Arabidopsis* and the Solanaceae.

All sequences of plant DAHP synthases translated from cDNAs have amino termini that are characteristic of signal sequences for plastid import, suggesting a plastidic location for the mature proteins. Transit sequences for chloroplast import are rich in hydroxylated amino acid residues and have a net positive charge but assume no particular secondary structure (Gavel and von Heijne, 1990). The putative transit sequences of all DAHP synthases described thus far fulfill these criteria, in confirmation of earlier work that indicated an intact shikimate pathway in the chloroplast. The most striking structural difference between the two DAHP synthase families specified by the *shkA* and *shkB* cDNAs is a deletion of approximately 20 amino acid residues close to the amino terminus in the *shkB* product (Fig. 3). Even though the *A. thaliana* sequences are quite different from those of the Solanaceae, in particular in the amino-terminal portion, the 20-residue deletion is positionally conserved for all three species. This structural difference may point to a difference in the final intracellular locale of the mature enzymes or to some difference in the efficiency of plastid import. The remaining sequence differences between the mature plant DAHP synthases are small, with no obvious hints about functional differences between the two families. The true functional role of the conserved 20-residue deletion has to remain a matter of speculation until further experimentation.

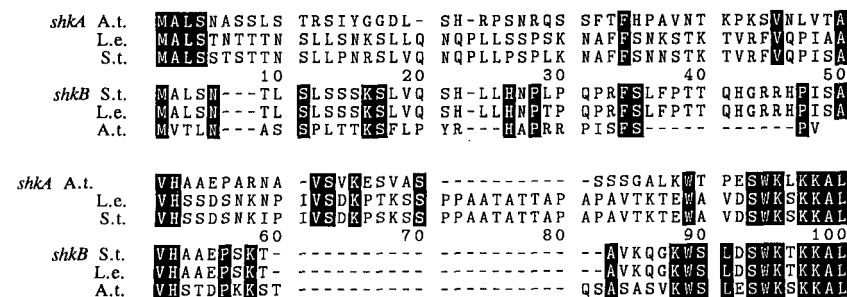
When the sequences of plant DAHP synthases are compared with those of their bacterial homologs, the identity scores decrease precipitously to the lower 20th percentile for pairs. When all known sequences are aligned using the program PRETTYBOX, only 24 invariant residues are seen. This low degree of identity was a matter of some concern during the structural analysis of the first plant DAHP synthase cDNA, because, at that time, other plant-bacteria homolog pairs showed at least 50% sequence identity at the protein level. Since then, functional complementation of yeast and *E. coli* mutants devoid of DAHP synthase has been demonstrated for the *A. thaliana* and the potato cDNA clones, respectively (Keith et al., 1991; Weaver et al., 1993). These experiments proved unequivocally the true nature of the DAHP synthase cDNAs.

Among the 24 amino acid residues that are invariant between all known DAHP synthase sequences is a Cys

residue that corresponds to Cys<sup>488</sup> of the *shkA* product and that is one of the two invariant Cys residues of the *E. coli* isoenzymes. Replacement of this residue in the *E. coli* enzyme with a different amino acid has a significantly negative effect on the  $V_{max}$  and the  $K_m$  of the enzyme, even though this Cys residue is not essential for catalytic activity (Stephens and Bauerle, 1992). The other invariant prokaryotic Cys residue is a metal ligand within the Cys-X-X-His motif mentioned above. This Cys residue is positionally not conserved when plant and bacterial enzymes are compared. However, like the bacterial enzymes, plant DAHP synthases are metalloproteins and contain one invariant Cys-X-X-His motif, residues 342 to 345 in the *shkA* product. The position of this Cys-X-X-His motif within the plant enzyme is quite interesting: it lies directly upstream of a fully conserved Gly-Glu-Arg-Thr sequence that could be part of the active site of the enzyme. The *E. coli* isoenzymes contain a conserved Gly-Ala-Arg-Thr sequence of active-site residues downstream and directly adjacent to residues that form part of the feedback inhibitor-binding site (Ray et al., 1988). Since none of the structurally characterized plant enzymes is feedback inhibited by any aromatic amino acid, one might speculate that the feedback inhibitor-binding site of prokaryotic enzymes is occupied by a metal-binding site in the plant enzyme. Future experiments involving site-directed mutagenesis will be necessary to test this hypothesis.

## REGULATION OF THE PLANT DAHP SYNTHASE

As the first enzyme of the shikimate pathway, the plant DAHP synthase would be a logical candidate for a regulatory protein subject to allosteric control by the aromatic amino acids as in bacteria. Unexpectedly, the purified enzymes from carrot and potato are activated by Trp and to a lesser degree by Tyr in a hysteretic fashion (Suzich et al., 1985; Pinto et al., 1986). Thus, the aromatic amino acids cannot be considered to be feedback inhibitors of plant DAHP synthases. It is reasonable to imagine that some other metabolite may regulate this enzyme at the protein level. Since 0.15 mM arogenate leads to 50% inhibition of the bean enzyme (Rubin and Jensen, 1985) and since the synthesis of this Phe and Tyr precursor is regulated by the intracellular concentrations of the three aromatic amino acids, Jensen (1986) proposed a model for sequential feedback inhibition of aromatic biosynthesis. This model would allow for cessation of carbon flow into the shikimate path-



**Figure 3.** Amino termini of *shkA*- and *shkB*-translated mRNA from potato (S.t.), tomato (L.e.), and *A. thaliana* (A.t.). Invariant residues to the potato *shkA* or *shkB* products, respectively, are highlighted; dashes indicate deletions.

way at high concentrations of aroenate, a rather unstable metabolic intermediate.

What about regulation at the mRNA level? If in plants the aromatic amino acids should not be considered primarily as destined for protein biosynthesis but rather as intermediates in the biosynthesis of secondary metabolites (Fig. 4), it is logical to ask whether any of these products regulate this enzyme. An alternative view is to consider DAHP synthase to be a housekeeping enzyme, constitutive in nature and not subject to regulation at the genetic level. The first hint of genetic control over the formation of plant DAHP synthase came from experiments with potato cell suspension cultures, in which chorismate synthesis was blocked by sublethal doses of glyphosate. Such cells respond to this block in the penultimate step of the shikimate pathway by increasing activity and amount of DAHP synthase severalfold within a few hours (Pinto et al., 1988). The corresponding mRNA also increases, suggesting a transcriptional control mechanism. The induction of DAHP synthase is specific, because the second enzyme of the shikimate pathway, dehydroquinate synthase, is not affected. Thus, *in vivo* inhibition of a late step in the pathway does not lead to a general induction of all of the enzymes but rather to a specific elevation of the first enzyme, the activity that controls carbon flow into the pathway.

*In vitro*, the herbicide has no effect on DAHP synthase activity. Although some of the details of the mechanism of the *in vivo* induction will become clear from promoter analysis, the question arises anew as to which small molecule serves as a signal. Do the aromatic amino acids serve as direct signals that trigger transcriptional control mechanisms or are the intracellular concentrations of some of the secondary metabolites critical determinants of the levels of DAHP synthase mRNA? Among the many secondary metabolites derived from the three aromatic amino acids is lignin, the second most abundant biopolymer. Lignin, the polyphenolic glue in cellulose fibers, not only imparts mechanical strength to all plants but also plays vital roles in plant defense against microorganisms, in wound healing,

in maintenance of structural integrity, and in water transport capacity. Mainly because of the material demands associated with lignin biosynthesis, a sizable proportion of all photosynthetically fixed carbon flows through the shikimate pathway at all times in all plants.

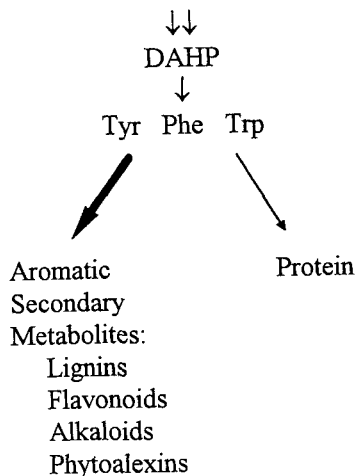
The rate of lignin biosynthesis can change rather abruptly, for example in wound repair. Indeed, DAHP synthase is a wound-inducible enzyme (Dyer et al., 1989; Keith et al., 1991). The induction is specific for *shkA*, whereas *shkB* is unaffected. The *shkA* promoter contains sequence elements that were identified previously in wound-inducible promoters. Thus, increased demand for lignin not only involves phenylpropanoid biosynthesis from Phe through induction of Phe ammonia lyase (Hahlbrock and Scheel, 1989) but also creates a signal for induction of DAHP synthase.

In addition, the *shkA* promoter contains sequence elements that are presumably target sites for visible light and UV light regulators, and light causes transcriptional induction of DAHP synthase in parsley cells in suspension culture (Henstrand et al., 1992). For both wound (Dyer et al., 1989) and light (Henstrand et al., 1992) induction, coordinate expression of DAHP synthase and Phe ammonia lyase suggests a tight coupling in the regulation of these two key enzymes in the synthesis of primary and secondary metabolites.

Such coordinate regulation may reflect the fact that changes in light conditions or in the demand for lignin can trigger general control mechanisms that affect many enzymes. Whether the biosynthesis of other specific secondary metabolites, for example anthocyanins, is likewise coordinately regulated at the transcriptional level is currently not known. In addition, a detailed understanding of the general induction of DAHP synthase awaits further experimentation.

#### DIFFERENTIAL EXPRESSION OF PLANT DAHP SYNTHASE ISOENZYMES

The two gene families encoding DAHP synthase respond differently to wounding and pathogen attack (Keith et al., 1991). Furthermore, there is a rather dramatic organ-specific expression of DAHP synthase isoenzymes (Görlach et al., 1993). The levels of *shkA* mRNA are much higher in stem and root than in leaf, flower, or cotyledon, where the majority of the DAHP synthase message is of the *shkB* type (Görlach et al., 1994). When this differential expression is compared with the abundance of mRNA for other shikimate pathway enzymes, one finds that the pattern of expression for the two DAHP synthases is different from the uniform pattern for the other enzymes (Görlach et al., 1994). The difference is consistent with the existence of a common transcriptional control for all but the first enzyme in the pathway. The fact that only one of the isoenzymes responds to wounding suggests different functions for the two identified enzyme families. There could be a highly regulatable function for the *shkA* product and its homologs, designed to satisfy a demand for secondary metabolites that can vary temporally as well as spatially and developmentally, in contrast to a basic housekeeping function for



**Figure 4.** The aromatic amino acids as intermediates in the biosynthesis of secondary metabolites.

the *shkB* product and its homologs geared to satisfy amino acid biosynthesis destined for protein production. This point of view is an updated version of the old dual-pathway hypothesis (Rothe et al., 1983; Jensen, 1986) with a new player and presumably at a different locale, since both the *shkA*- and *shkB*-encoded enzymes seem to be destined for plastid import and no cytosolic Mn<sup>2+</sup>-stimulated DAHP synthase has yet been identified.

## OUTLOOK

One focus of recent work from my laboratory has been the intracellular localization of DAHP synthase. The primary structures of these proteins, inferred from the cDNA sequences, point to a plastid localization for both isoenzyme families. Yet, by immunocytochemistry, most of the DAHP synthase cross-reacting material is found in the secondary cell wall of mature xylem vessel elements (Herrmann et al., 1991). If a major role for the enzyme is to provide precursors for lignin biosynthesis, one might expect to find this activity in the differentiating xylem, where lignin biosynthesis takes place. However, the secondary cell wall of fully differentiated xylem cells is not considered to be a very active cell compartment in the metabolic sense. It is tempting to speculate that DAHP synthase has two functions: a catalytic function as the first enzyme of lignin biosynthesis and a structural function in the lignified secondary cell wall. An alternative explanation is that a cell wall-localized protein shares an epitope with DAHP synthase. This protein may not have DAHP synthase activity but may be evolutionarily related to DAHP synthase.

Another tightly regulated enzyme of amino acid biosynthesis, Thr deaminase, which catalyzes the first committed step in Ile biosynthesis, is the major protein of floral parenchyma cells in tomato (Samach et al., 1991). The distribution of Thr deaminase within the plant or even within the flower does not reflect in vivo differences of Ile biosynthesis. Does this polypeptide have two different functions, an enzymatic function in Ile biosynthesis and a structural function in the terminally differentiated cell? If further experimental evidence for two such separate functions were obtained, both DAHP synthase and Thr deaminase could be added to the increasing list of proteins with multiple functions (Wistow, 1993). Such proteins may provide evidence for an evolutionary mechanism called gene sharing, which describes the possibility that a gene may acquire and maintain two functions without duplication (Piatigorsky et al., 1988). Current experimentation in my laboratory is exploring the possible dual function of plant DAHP synthase by analysis of transgenic plants.

The purification of carrot and potato DAHP synthase, the preparation of monospecific polyclonal antibodies against these enzymes, and the cloning of cDNAs encoding this activity have provided powerful tools to probe regulatory features in the synthesis of aromatic secondary metabolites and to analyze the temporal and spatial distribution of mRNA and protein. The elucidation of regulatory mechanisms for DAHP synthase expression and of the means by which the protein is transported to different intracellular locales promises to reveal novel aspects of

coordinate regulation and intracellular protein traffic in higher plants.

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