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The Shikimate Pathway: Early Steps in the Biosynthesis of Aromatic Compounds

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INTRODUCTION

The shikimate pathway was discovered as the biosynthetic route to the aromatic amino acids phenylalanine, tyrosine, and tryptophan through the classic studies of Bernhard Davis and David Sprinson and their collaborators. This pathway has been found only in microorganisms and plants. Phenylalanine and tryptophan are essential components of animal diets, and animals synthesize tyrosine in a single step from phenylalanine. Thus, with respect to plant specificity, the shikimate pathway is a bit more widespread than nitrogen fixation or photosynthesis but less ubiquitous than, for example, nitrogen assimilation.

Bacteria spend >90% of their total metabolic energy on protein biosynthesis. Consequently, the bacterial shikimate pathway serves almost exclusively to synthesize the aromatic amino acids (Herrmann, 1983; Pittard, 1987). In contrast, higher plants use these amino acids not only as protein building blocks but also, and in even greater quantities, as precursors for a large number of secondary metabolites, among them plant pigments, compounds to defend against insects and other herbivores (see Dixon and Paiva, 1995, this issue), UV light protectants, and, most importantly, lignin (Bentley, 1990; Singh et al., 1991; see Whetten and Sederoff, 1995, this issue). Under normal growth conditions, 20% of the carbon fixed by plants flows through the shikimate pathway (Haslam, 1993). Globally, this amounts to $\sim 7 \times 10^{15}$ kg each year, most of it used for the synthesis of the various secondary metabolites. And the variation in shikimate pathway–derived secondary metabolites is very extensive among plant species. The secondary metabolite makeup of a plant could be used for species classification.

Different plants not only synthesize different aromatic secondary metabolites but also synthesize varying amounts of them at specific times and in specific subcellular compartments. One would expect that regulation of the differential biosynthesis of sometimes very complex molecular structures might involve regulation of the supply of the precursors influencing the rate-limiting step for carbon flow through the shikimate pathway. Recent data on transgenic potatoes give some indication that this is indeed the case (Jones et al., 1995).

This review gives a short overview of the shikimate pathway and briefly introduces the seven enzymes that catalyze the sequential steps of the pathway. This is followed by a discussion of some enzymes of quinate metabolism, which use shikimate pathway intermediates as substrates, thus forming branches off the main trunk. I end by discussing some regulatory features of several of the enzymes.

THE SHIKIMATE PATHWAY

The biosynthesis of the three aromatic amino acids is best considered in two parts: the shikimate pathway from phosphoenol pyruvate and erythrose-4-phosphate to chorismate, which is common to phenylalanine, tyrosine, and tryptophan biosynthesis, and the three specific terminal pathways that use chorismate as a substrate. Because all three aromatic amino acids found in proteins are synthesized via the shikimate pathway, it has often been referred to as the common aromatic biosynthetic pathway (Herrmann, 1983). Bentley (1990) considers this notation incorrect because not all aromatic natural products originate with this metabolic sequence.

Figure 1 outlines the seven steps of the shikimate pathway. In the first step, the glycolytic intermediate phosphoenol pyruvate and the pentose phosphate pathway intermediate erythrose-4-phosphate are condensed to a seven-carbon six-membered heterocyclic compound, 3-deoxy-D-*arabino*-heptulosonate 7-phosphate (DAHP), which can formally be considered a 2-deoxy-D-glucose-6-phosphate derivative (Garner and Herrmann, 1984). In the second step, the ring oxygen is exchanged for the exocyclic C7 of DAHP to form a highly substituted cyclohexane derivative, 3-dehydroquinate. The remaining five steps serve to introduce a side chain and two of the three double bonds that convert this cyclohexane into the benzene ring, the hallmark of aromatic compounds. Figure 2 shows chorismate, the final product of the main trunk of the shikimate pathway, as a substrate for a number of anabolic sequences to primary and secondary compounds.

Figure 1 indicates the names of the seven enzymes of the shikimate pathway and the notation I propose for the plant genes encoding these enzymes. This gene notation is different from the bacterial *aro* mnemonic nomenclature. In response to the concerns mentioned by Bentley (1990), *shk* has been chosen to avoid potential conflicts with genes encoding enzymes of nonchorismate-derived aromatic biosynthesis. The

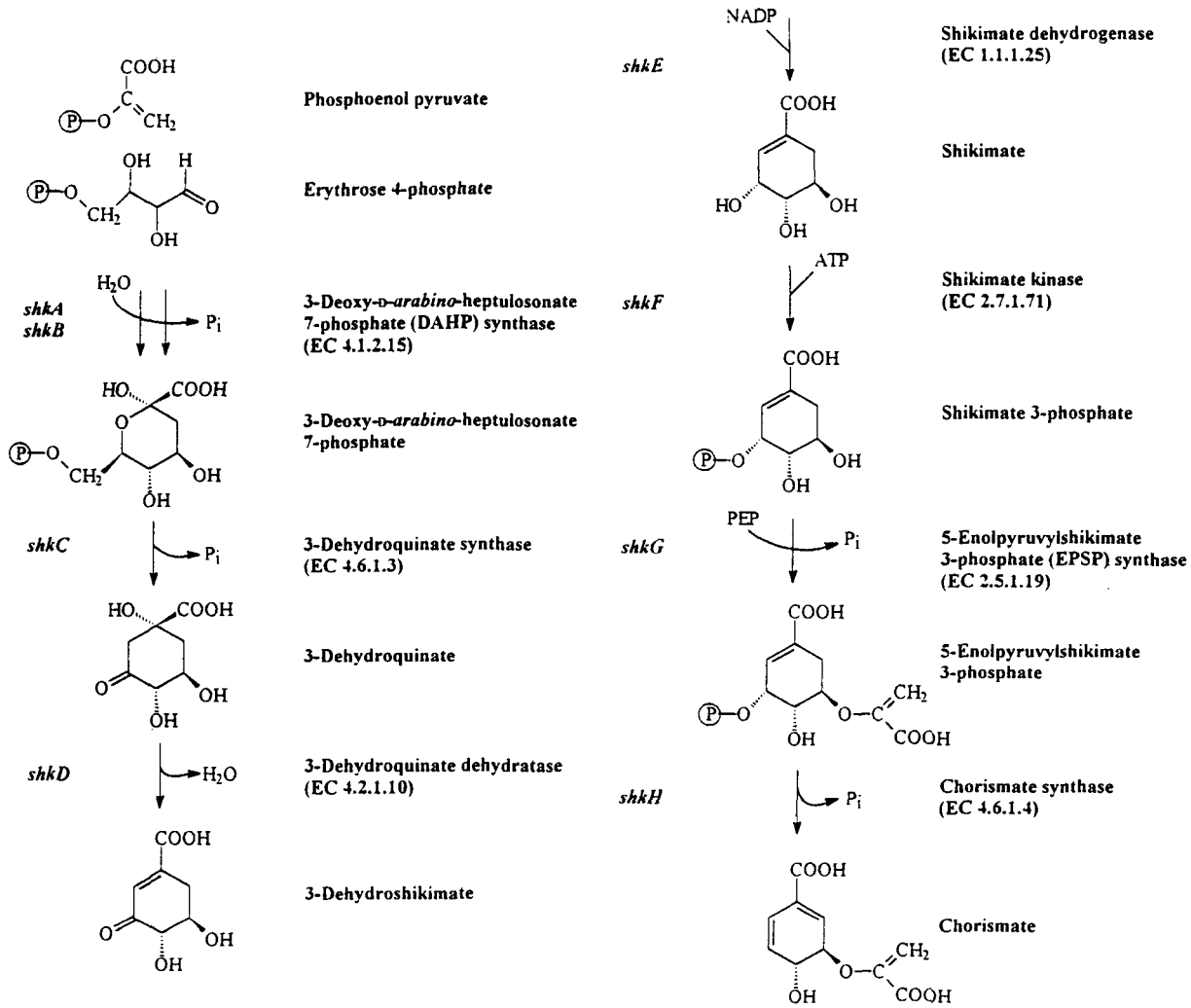

Figure 1. The Shikimate Pathway.

The abbreviations *shkA* through *shkH* are the notations proposed for the wild-type genes encoding shikimate pathway enzymes in higher plants.

designations *shkA* and *shkB* have been reserved for genes encoding isoenzymes that catalyze the first step, because these fall into two distinct families (Görlach et al., 1993a; Herrmann, 1995).

DAHP Synthase

The first committed step in the biosynthesis of the three aromatic amino acids is catalyzed by DAHP synthase. This enzyme has been purified to electrophoretic homogeneity from several microbial sources, and pure preparations of the carrot (Suzich et al., 1985) and potato (Pinto et al., 1986) enzymes have been prepared. The plant DAHP synthases are homodimeric enzymes activated by Mn^{2+} and tryptophan. Their subunit molecular weights of 53,000 to 55,000 are much larger than the corresponding values of the microbial homologs (Herrmann, 1995). Mn^{2+} and tryptophan are ligands that stabilize the quaternary structure of the enzymes in a hysteretic fashion. The K_m values for phosphoenol pyruvate and erythrose-4-phosphate are 30 and 70 μM, respectively, similar to the values obtained for DAHP synthases from microorganisms.

In contrast to the microbial enzymes, none of the plant DAHP synthases is inhibited by any of the three aromatic amino acids. *Escherichia coli*, for instance, has three DAHP synthase isoenzymes: one feedback inhibited by phenylalanine, one by tyrosine, and one by tryptophan. These isoenzymes, which are the products of three different genes, all require a metal ion for enzyme activity (McCandliss and Herrmann, 1978; Baasov and Knowles, 1989; Stephens and Bauerle, 1991) and

can be assayed independently because of their strict sensitivity to the individual amino acid (Herrmann, 1983). The number of isoenzymes and their sensitivities to the three aromatic amino acids vary from microbe to microbe and have been used to define taxa (Jensen, 1970; Ahmad et al., 1986).

The lack of feedback inhibition has made it difficult to distinguish between the plant isoenzymes. Early studies distinguished, on the basis of metal activation, a Mn^{2+}-dependent and a Co^{2+}-activated DAHP synthase (Rubin et al., 1982; Ganson et al., 1986). Several forms of the Mn^{2+}-stimulated enzyme have been separated by ion exchange chromatography and by chromatofocusing (Suzich et al., 1985; Pinto et al., 1986). These forms may be isoenzymes, because cloning efforts in several laboratories have identified two families of genes

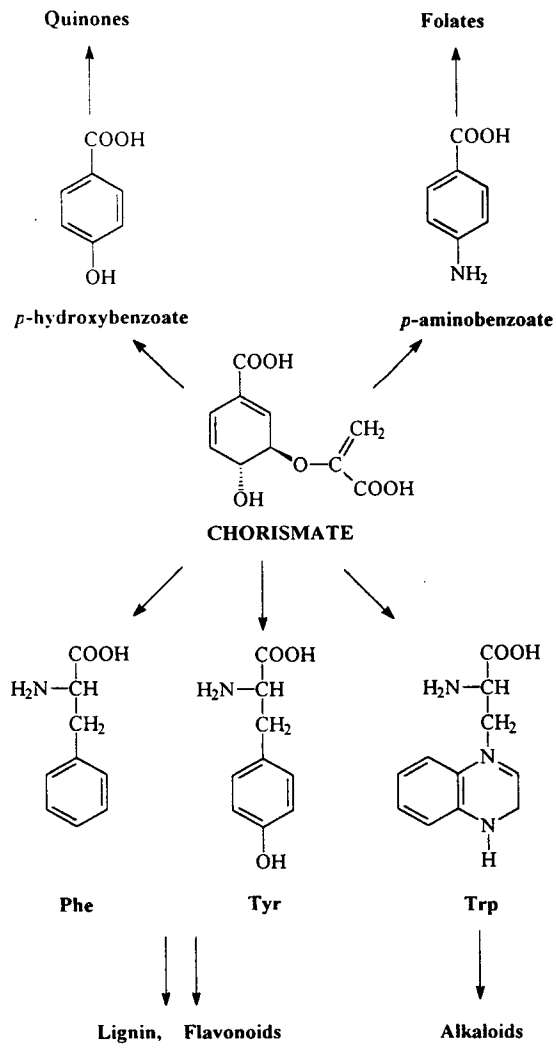

Figure 2. Chorismate as a Precursor for Primary and Secondary Metabolites.

The arrows indicate pathways and not necessarily single reactions. For example, conversion of chorismate to tryptophan requires five enzymes (see Radwanski and Last, 1995, this issue).

encoding Mn^{2+}-stimulated DAHP synthases, the *shkA* and *shkB* types. The *shkA*-type enzymes from different species resemble each other more than do the *shkA* and *shkB* enzymes from a single plant (Keith et al., 1991; Görlach et al., 1993a; Herrmann, 1995). The greater evolutionary distance between *shkA* and *shkB* suggests that DAHP synthase isoenzymes existed before genera such as *Solanum* and *Lycopersicon* diverged.

Recent studies of the Co^{2+}-activated enzyme have shown a broad substrate specificity with respect to the aldehydic substrate, and the Co^{2+}-activated enzyme has been found to have a K_m for erythrose-4-phosphate that is one order of magnitude higher than that of the Mn^{2+}-stimulated activity (Doong et al., 1992). Thus, the ability of the Co^{2+}-activated enzyme to supply DAHP for aromatic biosynthesis has been questioned (Görlach et al., 1993a). A careful molecular biological analysis and comparison of the amino acid sequences are required to determine how closely related this enzyme is to the known Mn^{2+}-stimulated DAHP synthases.

The first cDNA encoding a plant DAHP synthase (Dyer et al., 1990) was obtained from potato cells grown in suspension culture by screening a cDNA expression library with polyclonal antibodies against the potato tuber enzyme (Pinto et al., 1988). This potato cDNA was used to clone homologs from Arabidopsis (Keith et al., 1991), tobacco (Wang et al., 1991), and tomato (Görlach et al., 1993a) and a second cDNA from potato (Zhao and Herrmann, 1992). The deduced amino acid sequences of the plant enzymes show only ~20% identity with their microbial homologs. However, complementation of yeast (Keith et al., 1991) and *E. coli* (Weaver et al., 1993) mutants devoid of DAHP synthase with plasmids carrying the Arabidopsis and potato cDNAs, respectively, proved that those cDNAs encode polypeptides with DAHP synthase enzyme activity.

The plant cDNAs encode N termini that resemble transit sequences for chloroplast import (Gavel and von Heijne, 1990). Therefore, it seems likely that all of the Mn^{2+}-stimulated DAHP synthases reside in plastids. The Co^{2+}-activated DAHP synthase has been localized to the cytosol by discontinuous sucrose gradient centrifugation (Ganson et al., 1986). Because of the broad substrate specificity of this DAHP synthase, it remains a challenge to show, unequivocally, a specific enzyme dedicated exclusively to DAHP synthesis outside the plant chloroplasts.

3-Dehydroquinate Synthase

The second enzyme of the shikimate pathway, 3-dehydroquinate synthase, requires catalytic amounts of NAD^+ and a divalent cation for activity. For the bacterial enzyme, Co^{2+} and Zn^{2+} are the most active metal ions. The *E. coli* enzyme (Frost et al., 1984; Millar and Coggins, 1986) catalyzes a seemingly very complex reaction involving an intramolecular oxidation–reduction at C5 of DAHP with very tight binding of the NAD^+ cofactor, the *syn* elimination of phosphate, and an alicyclic ring formation (Bender et al., 1989). However, Widlanski et al. (1989)

have speculated that the enzyme may merely catalyze the oxidation and the reduction, with the other identified steps in the overall reaction mechanism proceeding spontaneously. The mung bean (Yamamoto, 1980) and pea (Pompliano et al., 1989) enzymes have been purified, but no cDNA encoding this enzyme has yet been isolated from any higher plant. 3-Dehydroquinate synthase is the only plant enzyme of the shikimate pathway for which no primary structural data are available.

3-Dehydroquinate Dehydratase•Shikimate Dehydrogenase

In higher plants, the third and fourth steps of the shikimate pathway are catalyzed by a bifunctional enzyme (Koshiba, 1978; Polley, 1978). In mung bean, the moss *Physcomitrella patens*, spinach, pea, and tobacco, the 3-dehydroquinate dehydratase and shikimate dehydrogenase activities reside on a single polypeptide (Fiedler and Schultz, 1985; Mousedale et al., 1987). Such bi- or even multifunctional enzymes are found in several amino acid biosynthetic pathways, but homologous reactions are sometimes structurally organized in different ways in different organisms. For example, in *Neurospora crassa* (Case and Giles, 1968), *Schizosaccharomyces pombe* (Nakanishi and Yamamoto, 1984), *Aspergillus nidulans* (Charles et al., 1986), and *Saccharomyces cerevisiae* (Duncan et al., 1987), the second through sixth reactions of the shikimate pathway are catalyzed by a pentafunctional polypeptide encoded by the *arom* gene. In contrast, in the bacteria *E. coli* and *Salmonella typhimurium*, the seven enzymes of the shikimate pathway are all individual polypeptides.

Fungi have two 3-dehydroquinate dehydratases with little apparent sequence homology (Giles et al., 1985). The type I enzyme is heat labile, catalyzes a *syn* elimination (Hanson and Rose, 1963), and has K_m values in the lower micromolar range. The type II enzyme is heat stable, catalyzes a *trans* elimination (Harris et al., 1993), and has K_m values one to two orders of magnitude higher than those of the type I enzyme. In general, the type II enzyme participates in 3-dehydroquinate degradation (see later discussion).

The 3-dehydroquinate dehydratases of most bacteria and higher plants are type I enzymes. In the *E. coli* 3-dehydroquinate dehydratase, a histidine residue (Deka et al., 1992) and a lysine residue (Chaudhuri et al., 1991) are in the active site of the enzyme. A type I enzyme has been crystallized from *S. typhimurium*; the crystals refract to 2.3-Å resolution (Boys et al., 1992). A type II 3-dehydroquinate dehydratase (Garbe et al., 1991) from *Mycobacterium tuberculosis* has been crystallized; the crystals refract to 2.2-Å resolution (Gourley et al., 1994). The analysis of these crystals will yield structures that may reveal some fundamental differences in the reaction mechanisms of enzyme-catalyzed *syn* and *anti* water elimination.

Several forms of the bifunctional 3-dehydroquinate dehydratase•shikimate dehydrogenase have been purified to electrophoretic homogeneity from the stroma of spinach chloroplasts (Fiedler and Schultz, 1985). The turnover number of 3-dehydroquinate dehydratase is approximately one-ninth that of shikimate dehydrogenase, so dehydroquinate is readily converted to shikimate without accumulation of dehydroshikimate. The level of dehydroshikimate is apparently kept low to prevent induction of the catabolic pathway to protocatechuate (see later discussion).

Partial cDNAs encoding the 3-dehydroquinate dehydratase•shikimate dehydrogenase of pea (Deka et al., 1994) and tobacco (Bonner and Jensen, 1994) have recently been cloned using antibodies against the pure bifunctional pea and tobacco enzymes. Although neither cDNA is full length, the more extensive tobacco sequence includes the entire coding region for the mature bifunctional enzyme plus 69 nucleotides upstream of this region. The latter were speculated to encode part of a transit peptide for plastid import. This putative transit peptide is rich in hydroxylated amino acid residues but has a net negative charge, a feature not yet seen for any other chloroplast transit peptide (Archer and Keegstra, 1990; Gavel and von Heijne, 1990). Pairwise sequence alignments of the two domains of the bifunctional tobacco dehydratase•dehydrogenase with the monofunctional homologs from other organisms show that the plant enzymes are evolutionarily closer to their prokaryotic than to their lower eukaryotic homologs. Amino acid sequence identities vary from 24 to 36%.

Shikimate Kinase

In the fifth step of the pathway, a kinase phosphorylates shikimate to yield shikimate 3-phosphate. Elimination of this phosphate two steps later leads to the second double bond of the benzene ring. *E. coli* has two shikimate kinases of similar size but with different K_m values for shikimate: ~5 mM for enzyme I, and 200 μM for enzyme II (Whipp and Pittard, 1995). Enzyme II may be the major activity for chorismate biosynthesis. The three-dimensional structure of shikimate kinase II was predicted by computer modeling based on adenylate kinase coordinates (Matsuo and Nishikawa, 1994). The synthesis of enzyme II is subject to regulation by the *tyr* and *trp* repressors; enzyme I is synthesized constitutively (Heatwole and Somerville, 1992).

Plant shikimate kinases have been described from pea (Mousedale and Coggins, 1985) and rice (Kavi Kishor, 1989), and the activity has been purified to near homogeneity from spinach chloroplasts (Schmidt et al., 1990). The first plant cDNA encoding shikimate kinase was isolated from tomato (Schmid et al., 1992) by performing the polymerase chain reaction using as primers two oligonucleotides whose sequences are conserved between genes for the *E. coli* (DeFeyter et al., 1986; Millar et al., 1986), *Erwinia chrysanthemi* (Minton et al., 1989), and *S. cerevisiae* (Duncan et al., 1987) enzymes. The deduced amino acid sequence of the tomato enzyme contains an N-terminal extension that resembles a transit sequence for chloroplast import. Indeed, in vitro–synthesized tomato shikimate kinase precursor is processed and taken up by isolated spinach chloroplasts (Schmid et al., 1992). DNA gel blots of tomato genomic

DNA are consistent with a single copy gene for shikimate kinase (Schmid et al., 1992). These data agree with earlier observations that spinach contains a single shikimate kinase localized exclusively to the chloroplasts (Schmidt et al., 1990).

EPSP Synthase

The penultimate step in the shikimate pathway is the reversible formation of 5-enolpyruvylshikimate 3-phosphate (EPSP) and inorganic phosphate from shikimate 3-phosphate and phosphoenolpyruvate. The reaction is catalyzed by EPSP synthase, the best studied of all the enzymes in this pathway. This enzyme has been purified from both microbial and plant sources (Duncan et al., 1984; Mousedale and Coggins, 1984). The enzyme-catalyzed reaction mechanism has been probed in great detail (Anderson et al., 1990), and arguments have been advanced for both ordered (Anderson and Johnson, 1990) and random (Gruys et al., 1993) kinetic mechanisms. Site-directed mutagenesis and nuclear magnetic resonance studies have placed a histidine residue very close to the active site of the enzyme (Shuttleworth and Evans, 1994). The *E. coli* enzyme was crystallized and the x-ray structure analyzed to 3-Å resolution (Stallings et al., 1991). This analysis shows that EPSP synthase has a two-domain structure, with the active site presumably near the interdomain crossover segment.

EPSP synthase is the unique target for the widely used broad-spectrum herbicide glyphosate (Steinrücken and Amrhein, 1980). Plant enzymes have K_i values for glyphosate that are approximately one order of magnitude lower than the values for the microbial enzymes. The inhibition is competitive with phosphoenolpyruvate. This metabolic intermediate is a substrate not only for EPSP synthase but also for a number of other enzymes of intermediary metabolism, including the first enzyme of the shikimate pathway. However, none of these other enzymes is inhibited by glyphosate. Glyphosate tolerance in plants has been obtained by expression of a mutant allele encoding a less sensitive bacterial enzyme (Stalker et al., 1985) or by overproduction of EPSP synthase (Smart et al., 1985; Shah et al., 1986; Goldsbrough et al., 1990).

The high degree of specificity of glyphosate for EPSP synthase has been explained by the fact that the herbicide binds to the protein only in close proximity to shikimate 3-phosphate (Christensen and Schaefer, 1993). However, the product of the enzyme-catalyzed reaction, EPSP, also facilitates glyphosate binding; although the exact spatial orientation between glyphosate and the enzyme in the ternary complexes with shikimate 3-phosphate or EPSP is unknown, part of glyphosate must bind near to but outside the active site (Sammons et al., 1995).

Isoenzymes of EPSP synthase with similar K_m and K_i values for glyphosate have been separated by HPLC from seedling extracts of *Sorghum bicolor* (Ream et al., 1988), and molecular biological data are consistent with isoenzymes in other plants as well. For example, two forms of the maize enzyme have been purified to homogeneity that differ in thermal stability and regulatory properties but that are both localized to plastids (Forlani et al., 1994).

Plant DNAs encoding EPSP synthase have been isolated from petunia (Shah et al., 1986), Arabidopsis (Klee et al., 1987), tomato (Gasser et al., 1988), and *Brassica napus* (Gasser and Klee, 1990). All the cDNAs encode precursor proteins with N-terminal transit sequences for plastid import. Amino acid sequence comparison of the different plant enzymes shows ~90% identity in the mature proteins. Amino acid sequences of the mature plant EPSP synthases are more similar to homologs from bacteria than to those from fungi. In vitro uptake experiments have shown that the petunia transit sequence directs import into plastids (Della-Cioppa et al., 1986), and immunocytochemistry on suspension-cultured plant cells confirms the plastidic locale of the enzyme in vivo (Smart and Amrhein, 1987). Glyphosate inhibits the uptake of the enzyme into the plastid; efficient translocation apparently requires the unfolding of the polypeptide, a process that may be prevented or severely inhibited by the herbicide (Della-Cioppa and Kishore, 1988). To what extent this lack of efficient translocation contributes to the herbicidal effect of glyphosate is unclear, because tight binding of the herbicide to the enzyme requires shikimate 3-phosphate or EPSP, metabolic intermediates that may reside exclusively in the plastids.

Chorismate Synthase

The seventh and last step of the shikimate pathway is the concerted 1,4-*trans* elimination of phosphate from EPSP to yield chorismate (Balasubramanian et al., 1990; Hawkes et al., 1990). Chorismate synthase, which catalyzes this reaction, requires a reduced flavin nucleotide ($FMNH_2$) as a cofactor, even though the overall reaction is redox neutral. The same is true of step two of the pathway, which is catalyzed by 3-dehydroquinate synthase, an enzyme that requires NAD^+ for catalytic activity. Spectroscopic analysis of cofactor function suggested a radical as an intermediate in the reaction catalyzed by chorismate synthase (Ramjee et al., 1992). The *N. crassa* (Welch et al., 1974) and *Euglena gracilis* (Schaller et al., 1991b) enzymes are bifunctional; an associated NADPH-driven flavin reductase generates the reduced cofactor.

In contrast to the fungal enzymes, *E. coli* (White et al., 1988) and higher plant enzymes (Schaller et al., 1990) are monofunctional and require $FMNH_2$ and strictly anaerobic assay conditions. As noted by Schaller et al. (1991a), the difference between the monofunctional and bifunctional enzymes, that is, the potential lack of the flavin reductase domain in the monofunctional enzyme, is also reflected in the subunit sizes of the oligomeric enzymes. In *B. subtilis*, the reductase is a separate polypeptide that is part of a trifunctional enzyme chorismate synthase•3-dehydroquinate synthase•flavin reductase (Hasan and Nester, 1978). The two enzymes of the pathway that require redox cofactors in overall redox-neutral reactions are physically associated, even though they do not catalyze consecutive steps in this metabolic sequence.

Plant chorismate synthase was first demonstrated in pea chloroplasts (Mousedale and Coggins, 1986) and purified to homogeneity from cells of *Corydalis sempervirens* (Schaller et al., 1990). The first plant cDNA encoding chorismate synthase was isolated by expression cloning using a polyclonal antibody raised against this enzyme (Schaller et al., 1991a). This cDNA and two tomato homologs (Görlach et al., 1993b) encode polypeptides with N-terminal extensions that resemble transit peptides for chloroplast import.

QUINATE DEGRADATION

At least two intermediates of the biosynthetic pathway toward chorismate, 3-dehydroquinate and 3-dehydroshikimate, are also intermediates in the degradation of quinate. Figure 3 shows this metabolite as a precursor of chlorogenate, which accumulates in several plants (for example, coffee bean), to substantial levels. Although the accumulation of quinate appears to be restricted to specific plants, the occurrence of chlorogenate and its derivatives is more widespread. Chlorogenate is the major soluble phenylpropanoid in tobacco and a preformed protectant against fungal attack (Maher et al., 1994). It contributes to general plant health as part of a physical and possibly also a chemical barrier against microbial attack. Once this barrier is broken, the constituents become potential carbon sources for the attacker.

During a careful analysis of fungal mutants with lesions in the genes encoding biosynthetic shikimate pathway enzymes, Giles et al. (1985) detected a catabolic pathway for quinate, which, along with shikimate, is readily degraded by fungi and some bacteria, first to protocatechuate and ultimately to succinate and acetyl-CoA. In fact, *N. crassa* (Giles et al., 1985), *A. nidulans* (Grant et al., 1988), and *Rhodococcus rhodochrous* (Bruce and Cain, 1990) can all use quinate (or shikimate) as their only carbon source.

The first three steps of quinate catabolism are an NAD-dependent oxidation to 3-dehydroquinate followed by two dehydrations, first to 3-dehydroshikimate and then to protocatechuate. The enzymes catalyzing these reactions are quinate dehydrogenase, 3-dehydroquinate dehydratase, and 3-dehydroshikimate dehydratase, respectively. Their primary structures have been deduced from the corresponding gene sequences (Giles et al., 1985; Grant et al., 1988). The quinate dehydrogenase also oxidizes shikimate to 3-dehydroshikimate. This NAD-dependent enzyme activity of a shikimate dehydrogenase is different from that of the anabolic NADP-dependent isoenzyme. Catabolic and anabolic shikimate dehydrogenases are structurally different, even though they share some sequence similarities (Anton and Coggins, 1988). Most but not all catabolic quinate dehydrogenases are NAD dependent; one exception is quinate dehydrogenase of *Acinetobacter calcoaceticus*, which oxidizes quinate and shikimate to their 3-dehydro derivatives (Kleef and Duine, 1988) using as a cofactor pyrrolo-quinoline quinone (Duine and Jongejan, 1989; Elsemore and Ornstrom, 1994).

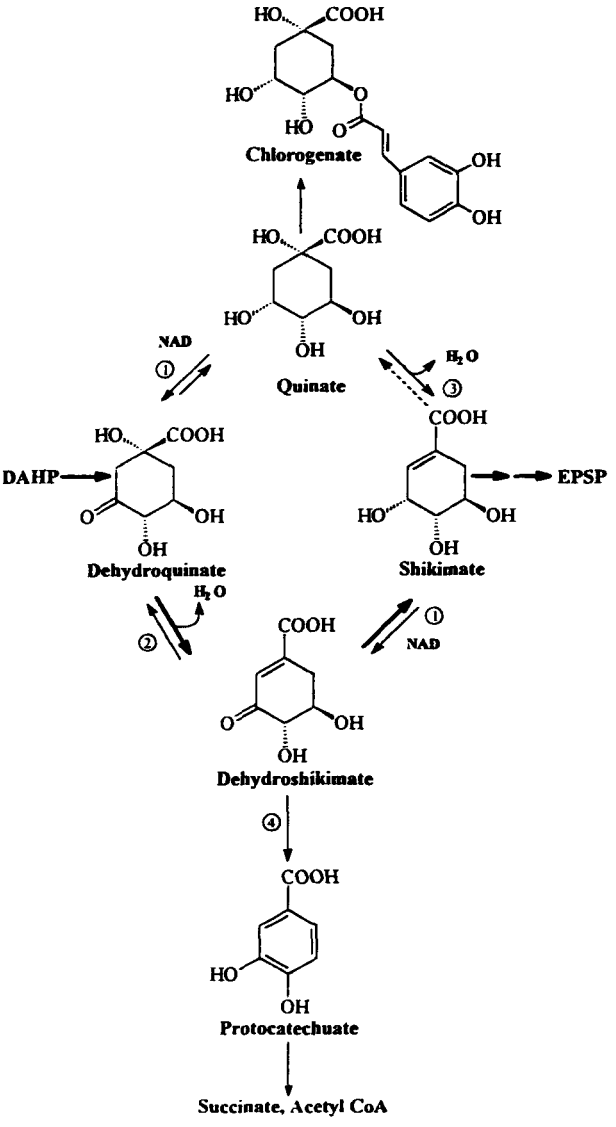

Figure 3. Metabolic Intermediates of the Shikimate Pathway in Quinate Synthesis and Degradation.

The enzymes of the quinate cycle are (1) quinate (shikimate) dehydrogenase; (2) 3-dehydroquinate dehydratase; and (3) quinate hydrolyase. 3-Dehydroshikimate is aromatized to protocatechuate by 3-dehydroshikimate dehydratase (4). Bold arrows indicate reactions of the main trunk of the shikimate pathway.

The second enzyme of fungal quinate degradation, the 3-dehydroquinate dehydratase, is also structurally different from the biosynthetic isoenzyme, which is encoded by part of the *arom* cluster described previously. The third enzyme in this catabolic sequence, the 3-dehydroshikimate dehydratase, catalyzes a *syn* elimination of water to yield protocatechuate (Scharf et al., 1971). Thus, fungi have clearly separated pathways for the biosynthesis of the aromatic amino acids and the

degradation of quinate, even though 3-dehydroquinate and 3-dehydroshikimate are metabolic intermediates in both.

Whereas fungi use quinate as a carbon source for growth, in higher plants, quinate is the precursor for chlorogenate and may also serve as a storage or transport form of carbon (Beaudoin-Eagan and Thorpe, 1984). Quinate could be synthesized in one step from dehydroquinate by quinate dehydrogenase or from shikimate by quinate hydrolyase (Figure 3). Plants can also use quinate as a carbon source for aromatic amino acids (Leuschner and Schultz, 1991a, 1991b). Whether the two enzymes, the dehydrogenase and the hydrolyase, serve in both an anabolic and a catabolic capacity or whether one is used for quinate biosynthesis and the other for quinate degradation remains an open question.

Plant quinate dehydrogenase was discovered in bean cells (Gamborg, 1966) and purified to homogeneity from carrot cells (Refeno et al., 1982) and mung beans (Kang et al., 1994). The bean enzyme is localized to the plastid stroma and can use both NAD and NADP as cofactors.

A quinate hydrolyase has recently been isolated from pea roots (Leuschner et al., 1995). This plastid-localized enzyme converts quinate directly to shikimate, a reaction envisioned more than 100 years ago by the discoverer of shikimate (Eykmann, 1891). Together with quinate dehydrogenase and dehydroquinate dehydratase, quinate hydrolyase forms what is shown in Figure 3 as the quinate cycle. Identification of these three enzymes as anabolic, catabolic, or both remains to be made.

REGULATION OF CARBON FLOW IN THE SHIKIMATE PATHWAY

All enzymes of the shikimate pathway have been obtained in pure form, and some of their properties have been described. The primary structures are known for six of the seven higher plant activities in the main trunk of this metabolic sequence. For both plants and bacteria, at least two of the seven enzymes can be regulated. Regulatory features have been elucidated for DAHP synthase and shikimate kinase.

Regulation of the shikimate pathway in lower eukaryotes is different from that in prokaryotes and involves feedback inhibition and both transcriptional and translational control (Braus, 1991). DAHP synthase isoenzymes that are sensitive to specific aromatic amino acids are similar to their prokaryotic homologs with respect to substrate and inhibitor binding constants. However, control of shikimate pathway gene expression in yeast and fungi is very different from that in prokaryotes. In yeast, for example, the transcriptional activator GCN4 controls initiation of transcription for ~30 genes encoding enzymes of several amino acid biosynthetic pathways, including those for the aromatic amino acids. Starvation for any one amino acid leads to derepression of all the genes. GCN4 affects RNA polymerase II activity by binding to DNA recognition elements that have been identified in all GCN4-controlled promoters, including those for genes encoding DAHP synthase and chorismate synthase. The expression of GCN4 itself is under translational control that depends on several other positive effectors, including the protein kinase GCN2.

The prokaryotic DAHP synthase is regulated at two levels. Feedback inhibition of enzyme activity and transcriptional control of enzyme synthesis are mediated by the three aromatic amino acids and affect specific isoenzymes. The genes encoding the tyrosine- and tryptophan-sensitive isoenzymes are regulated by the *tyr* and *trp* repressors, respectively (Garner and Herrmann, 1985; Klig et al., 1988), and the synthesis of the tryptophan-sensitive isoenzyme is affected by both repressors (Muday et al., 1991). However, feedback inhibition appears to be the major regulatory mechanism in vivo (Ogino et al., 1982).

In contrast with bacterial and lower eukaryote DAHP synthases, plant DAHP synthases are not subject to feedback inhibition by the aromatic amino acids; in fact, both tyrosine and tryptophan act as activating ligands of the enzymes from carrot and potato by affecting the quaternary structures of these catalysts (Suzich et al., 1985; Pinto et al., 1986). The first evidence of metabolic regulation of a plant DAHP synthase came from experiments with suspension-cultured potato cells exposed to glyphosate (Pinto et al., 1988). In vivo, this herbicide reduces chorismate synthesis by inhibiting the penultimate step in the pathway (see earlier discussion). In addition, it increases both the activity and amount of DAHP synthase. We know this increase is specific, because the activity of 3-dehydroquinate synthase, the second enzyme in the pathway, is unaffected. The herbicide has no effect on DAHP synthase in vitro, however, indicating that inhibition of chorismate synthesis results in the production of a signal that presumably affects transcription or translation of the gene encoding DAHP synthase. The nature of this signal is unknown.

Stress on intact plants has similar effects on DAHP synthase levels. Both mechanical wounding (Dyer et al., 1989) and fungal elicitation (McCue and Conn, 1989; Keith et al., 1991; Görlach et al., 1995) induce DAHP synthase mRNA accumulation. Thus, increased carbon flow into the shikimate pathway in response to different environmental demands appears to be accomplished by derepression of DAHP synthase. This derepression is specific for isoenzymes of the *shkA* type (Keith et al., 1991; J. Zhao, M. Sakuta, L.M. Weaver, and K.M. Herrmann, unpublished results).

When plants are mechanically wounded or challenged by microbial or insect attack, much of the increased demand for chorismate may be for lignin to repair the lesions. DAHP synthase apparently plays a major control function in lignin biosynthesis, because transgenic plants that express *shkA*-type DAHP synthase antisense RNA are impaired in lignin biosynthesis (Jones et al., 1995).

Expression of DAHP synthase not only is affected by environmental stimuli but also is tissue specific, and mRNA levels of all but the first enzyme of the pathway appear to be regulated in concert (Görlach et al., 1994). In tomato, similar patterns have been found for organ-specific mRNA accumulation of shikimate kinase, EPSP synthase, and chorismate synthase.

The levels of mRNA encoding DAHP synthase isoenzymes differ from each other and from mRNA levels of the other shikimate pathway enzymes.

Any consideration of the regulation of a plant pathway should include the subcellular locale of the metabolic sequence. Isolated chloroplasts incorporate $^{14}CO_2$ into aromatic amino acids, and enzymes of the shikimate pathway can be assayed in preparations of isolated chloroplasts (Bickel and Schultz, 1979; Schulze-Siebert et al., 1984; Fiedler and Schultz, 1985; Mousedale and Coggins, 1985). Schmid and Amrhein (1995) have advanced a different convincing argument for chorismate biosynthesis in chloroplasts, which is that all cDNAs so far isolated for enzymes of the shikimate pathway encode N-terminal extensions of the native polypeptides with characteristics of transit peptides for chloroplast import. Moreover, when synthesized in vitro, several of the precursor proteins are processed by and imported into isolated chloroplasts. Finally, when the amino acid sequences of these enzymes from different organisms are compared, in general greater similarities occur between plant and bacterial homologs than between plant and fungal homologs. This may reflect the endosymbiotic hypothesis of chloroplast evolution (Gray and Doolittle, 1982). Thus, it is reasonable to assume that at least some, if not all, chorismate biosynthesis occurs in plastids. Even though all the enzymes of this pathway are synthesized on cytosolic ribosomes and some unprocessed precursors are enzymatically active (Schmid et al., 1992), and even though great efforts have been expended to demonstrate cytosolic aromatic amino acid biosynthesis, conclusive evidence for a complete and active extraplastidial shikimate pathway is still outstanding (Forlani et al., 1994).

Shikimate kinase may represent a secondary control in the shikimate pathway and one of importance for secondary metabolism. One of the two *E. coli* shikimate kinase isoenzymes is synthesized constitutively, and the synthesis of the other is under control of the *tyr* and *trp* repressors (Ely and Pittard, 1979; DeFeyter and Pittard, 1986; Heatwole and Somerville, 1992; Whipp and Pittard, 1995). Possibly more pertinent to plants is the observation that plant shikimate kinase is subject to control by energy charge (Schmidt et al., 1990). Such a control could indicate that in plants, under energetically favorable conditions, carbon of shikimate is diverted from the main trunk of the pathway into secondary products or stored in the form of quinate and its derivatives. Little else is known about the regulation of quinate synthesis.

Quinate degradation is also subject to regulation. In lower eukaryotes, the three enzymes of quinate breakdown to protocatechuate are induced by quinate. This induction is governed by two regulatory proteins, a transcriptional activator and a transcriptional repressor. The two genes encoding these regulatory proteins appear to have evolved by splitting an early anabolic *arom* gene (Hawkins et al., 1993), giving an intriguing glimpse into the evolution of fungal regulatory circuits.

In higher plants, quinate can also be converted back into intermediates of the shikimate pathway either by oxidation to 3-dehydroquinate or by dehydration directly to shikimate (Figure 3). Nothing is known about the regulation of the quinate dehydratase in plants. However, the activity of quinate dehydrogenase is regulated by reversible Ca^{2+}-modulated protein phosphorylation at a serine residue (Ranjeva et al., 1983; Graziana et al., 1984; Ranjeva and Boudet, 1987). This modulation of quinate dehydrogenase, coupled with the recent finding of voltage-activated Ca^{2+} channels in plant plasma membranes (Thuleau et al., 1993, 1994), may announce the end of the era in which quinate was "a Cinderella still awaiting a ticket to the ball" (Haslam, 1993); the Cinderella may be slipping into her shoes right now.

OUTLOOK

The shikimate pathway is one of the major biosynthetic pathways in higher plants. In excess of one-fifth of all fixed carbon flows through this metabolic sequence. All enzymes of the shikimate pathway have been isolated and their kinetic parameters characterized. Some of the reaction mechanisms have been studied in detail. The primary structures of all but one of the enzymes have been obtained through the combined efforts of protein and DNA sequencing, and the first three-dimensional structures are appearing. The seven reactions of the pathway are identical in bacteria, eukaryotic microorganisms, and plants. The plant enzymes seem more closely related evolutionarily to their bacterial than to their fungal homologs.

The available information is insufficient to make a similar statement about the regulation of this pathway. Some regulatory mechanisms have been described, and they differ substantially between these groups of organisms. Carbon flow into the bacterial and fungal shikimate pathway is controlled by feedback inhibition of aromatic amino acid–sensitive DAHP synthase isoenzymes. Plant DAHP synthases are not inhibited by the aromatic amino acids.

Genetic control of the bacterial shikimate pathway is at the transcriptional level by aromatic amino acid–mediated repression. The pathway in yeast is under GCN4-mediated general amino acid control, a combined transcription–translation activation. Plant DAHP synthase mRNA levels reflect environmental stimuli. However, neither transcriptional repression nor transcriptional or translational activation of gene expression has yet been described. Promoter analysis has revealed DNA motifs for wound induction and tissue-specific expression (L.M. Weaver, J. Zhao, and K.M. Herrmann, unpublished data), but *trans*-acting factors have so far been elusive.

Mutants lacking one of the DAHP synthase isoenzymes have not yet been described. In addition, a major step forward in our understanding of the mechanism of wound- and elicitor-induced elevation of DAHP synthase will come with the isolation of mutants with lesions in this signal transduction pathway.

One intracellular locale of the shikimate pathway is the plastid, but it remains to be seen whether this is the only site of chorismate biosynthesis. Using immunocytochemistry, DAHP synthase cross-reacting material is found in the secondary cell

wall of mature xylem vessel elements (Herrmann et al., 1991). This finding suggests two functions for this protein: a catalytic function as the first enzyme in lignin biosynthesis and a structural function in the lignified secondary cell wall.

Such dual functions of polypeptides may not be uncommon. The petunia gene encoding EPSP synthase is expressed very highly in flower petals and much less in leaves and other tissues. Deletion analysis of a petunia EPSP synthase promoter in transgenic petunia plants identified tissue-specific *cis*-regulatory sequences that are ~800 bp upstream of the transcription start site (Benfey et al., 1990). These regulatory sequences are larger than 500 bp and consist of several widely separated elements. Further evidence of high-level EPSP synthase expression in flowers comes from the finding that when the EPSP synthase promoter was used to drive the expression of the maize *A1* gene, which encodes an enzyme of anthocyanin biosynthesis, the resulting transgenic petunia plants showed pink flower petals, in contrast with colorless petals of the parental line. The significance of high levels of EPSP synthase in flower petals is not obvious. Possibly, the polypeptide serves a structural function as well as an enzymatic function.

A third example of a dual function plant protein appears to be threonine deaminase. This enzyme catalyzes the first step in isoleucine biosynthesis (see Singh and Shaner, 1995, this issue), but it is also the major protein of floral parenchyma cells in tomato (Samach et al., 1991). Again, this protein may have two functions: an enzymatic function in isoleucine biosynthesis, and a structural function in a terminally differentiated cell.

Additional experimental evidence is necessary before DAHP synthase, EPSP synthase, and threonine deaminase can be firmly established as dual function proteins analogous with those that have been described in a number of other eukaryotic systems (Wistow, 1993). Thus, future studies on the shikimate pathway not only may identify signals from secondary products that affect control points in primary metabolic pathways but also may contribute basic insights into processes of intracellular protein trafficking. In the coming years, experimentation on the shikimate pathway can be expected to have a major impact on these two research areas unique to plants.

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