

CHAPTER 3

THE BIOSYNTHESIS OF FLAVONOIDS

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1. INTRODUCTION

Flavonoids have long sparked the interest of scientists and nonscientists alike, largely because these metabolites account for much of the red, blue, and purple pigmentation found in plants and increasingly for their association with the health benefits of wine, chocolate, and generally with diets rich in fruits and vegetables. The flavonoid pathway, illustrated in Figure 3.1, therefore has become one of the most well-studied of the many unique secondary metabolic systems that characterize the plant kingdom. There is good evidence that these systems are derived from primary metabolism, with a variety of enzymes, including members of the cytochrome P450 hydroxylase, 2-oxoglutarate-dependent dioxygenase (2-ODDs), short-chain dehydrogenase/reductase (SDR), *O*-methyltransferase (OMT), and glycosyltransferase (GT) families, having been recruited into new functions during the rapid evolution that accompanied the movement of plants onto land (Stafford, 1991). In the case of flavonoids, rudimentary forms of these compounds may have played early roles as signaling molecules and then evolved functions in processes as diverse as UV protection, growth and development, defense against herbivores and pathogens, and recruitment of pollinators and seed dispersers. The remarkable diversity of form and function of flavonoids in present-day plants has provided a rich foundation for research in areas ranging from genetics and biochemistry to chemical ecology and evolution to human health and nutrition. To date, more than 6,400 different flavonoid compounds have been described in the literature (Harborne and Baxter, 1999) and the pathways responsible for their synthesis have been characterized in detail in numerous plant species (Dixon and Steele, 1999; Harborne and Williams, 2000, 2001; Winkel-Shirley, 2001a; Springob et al., 2003).

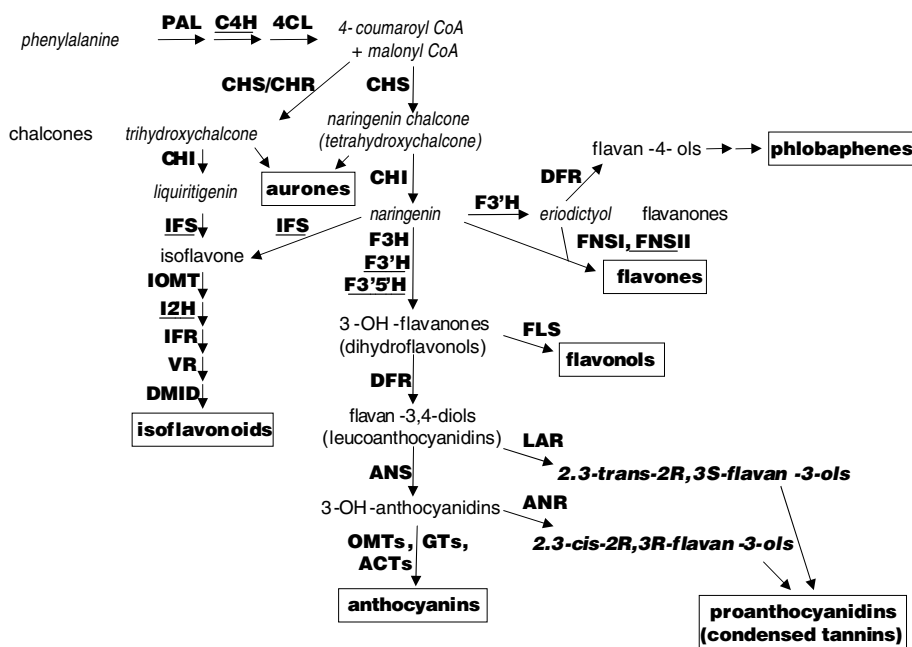


Figure 3.1 Schematic of the flavonoid pathway showing the enzymatic steps leading to the major classes of end products, flavonols, anthocyanins, proanthocyanidins, phlobaphenes, aurones, flavones, and isoflavonoids, which are identified with boxes. Names of the major classes of intermediates are given, with names of specific compounds in italics. Enzymes are indicated with standard abbreviations in bold; names of cytochrome P450 monooxygenases that may function as membrane anchors for other flavonoid enzymes are underlined. Abbreviations: ACTs, acetyl transferases; ANR, anthocyanidin reductase; ANS, anthocyanidin synthase (also known as leucoanthocyanidin dioxygenase); C4H, cinnamate-4-hydroxylase; CHI, chalcone isomerase; CHR, chalcone reductase; CHS, chalcone synthase; 4CL, 4-coumaroyl:CoA-ligase; DFR, dihydroflavonol 4-reductase; DMID, 7,2'-dihydroxy, 4'-methoxyisoflavanol dehydratase; F3'H, flavanone 3-hydroxylase; FNSI and FNSII, flavone synthase I and II; F3'5'H, flavonoid 3' and 3'5' hydroxylase; IOMT, isoflavone O-methyltransferase; IFR, isoflavone reductase; I2'H, isoflavone 2'-hydroxylase; IFS, isoflavone synthase; LAR, leucoanthocyanidin reductase; OMTs, O-methyltransferase; PAL, phenylalanine ammonia-lyase; GTs, glucosyl transferases; VR, vestitone reductase.

There also is a growing understanding of the diverse physiological functions of these compounds in plants and their effects, both beneficial and detrimental, when consumed by mammals. The aim of this chapter is to provide a brief historical account of the work that has led to our current understanding of the flavonoid pathway and then discuss recent advances in elucidating the biochemistry and organization of this intriguing metabolic system.

2. A BRIEF HISTORY OF RESEARCH ON FLAVONOID BIOSYNTHESIS

In-depth historical accounts of research leading to our current understanding of the structure and synthesis of flavonoid pigments can be found in two classic texts, Muriel Wheldale Onslow's *The Anthocyanin Pigments of Plants*, 2nd ed. (Onslow, 1925) and Helen Stafford's *Flavonoid Metabolism* (Stafford, 1990) (Figure 3.2), highlights of which are recounted here. Among the earliest documented studies of flavonoid pigments is Robert Boyle's 1664 *Experiments and Considerations Touching Colours* (Boyle, 1664), which describes the effects of acids and bases on the color of extracts from plant flowers and other pigmented tissues. The chemical compositions of flavonoids, specifically the blue pigment of cornflower and the red pigment of wine, was described in the mid-1800s as involving carbon, hydrogen, and oxygen (Morot, 1849; Glénard, 1858). Colorless substances also began to be recognized as being related to anthocyanin pigments during this period (Filhol, 1854; Wigand, 1862).

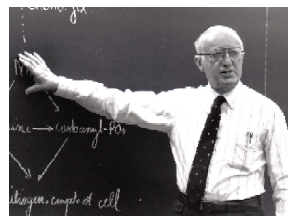
The first major progress toward understanding the biochemistry and genetics of flavonoid metabolism came from studies of inheritance, starting with Mendel's use of flower color in pea to study the segregation of visible traits (Mendel, 1865), and with renewed focus in the early 1900s in studies with common stock (a close relative of *Arabidopsis thaliana*) and sweet pea by Bateson, Saunders, and Punnett (1904, 1905) (Figure 3.2). The finding that purple-flowered progeny could be produced by crossing different white-flowered lines gave rise to the hypothesis that two genetic factors, *C* and *R*, were required for the production of red pigments in sweet pea, which were further modified by a third factor, *B*, producing blue or purple pigments. These critical insights opened the door to elucidating the chemical processes that underlie flavonoid biosynthesis. Palladin's theory of "Atmuhngspigmente," which proposed that plants contain chromogens that are oxidized by enzymes to pigments, including anthocyanins (Palladin, 1908), together with chemical and genetic studies in *Antirrhinum*, led Wheldale to suggest that anthocyanins are formed from a flavanone by the action of oxidase and reductase enzymes (Wheldale, 1909a, 1909b). Substantial progress was made soon thereafter on the chemical structures of flavonoids by Willstätter and colleagues, who showed that pigments from a wide range of plant species all derived from the three anthocyanins: pelargonidin, cyanidin, and delphinidin (e.g., Willstätter and Everest, 1913; Willstätter and Weil, 1916). This group also described the chemical relationship of anthocyanins with the flavonols, quercetin, kaempferol, and myricetin, as well as the presence of sugar and methoxy groups on these compounds.



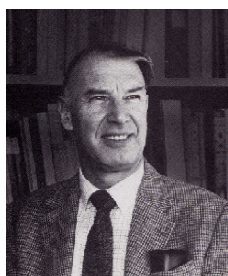
A. Reginald Punnett (1875-1967) and William Bateson (1861-1926) in 1907.



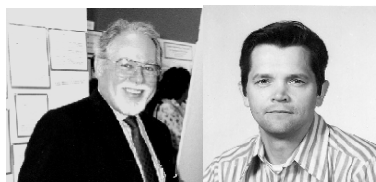
B. Helen Stafford in her laboratory at Reed College, 1960.



C. Eric Conn lecturing on nitrogen metabolism in the General Biochemistry course at Davis.



D. Hans Grisebach (1926-1990).



E. Geza Hrazdina and George Wagner, early 1990s.



F. Klaus Hahlbrock with Fritz Kreuzaler at his Ph.D. examination, ~1974.

Figure 3.2 Some of the key contributors to research on flavonoid metabolism. Photographs reprinted with permission from Mary Catherine Bateson (A); courtesy of Special Collections, Eric V. Hauser Memorial Library, Reed College (B); and with permission from the Phytochemical Society (C); photographs provided by Eric Conn (D) and Geza Hrazdina and George Wagner (E). Reprinted with permission from Mary Catherine Bateson, Institute for Intercultural Studies.

However, it was not until the 1950s that the availability of radioisotope tracer molecules fueled major new progress toward elucidating the flavonoid biosynthetic pathway (reviewed in Stafford, 1990). These studies also generated the first evidence for channeling of intermediates in phenylpropanoid biosynthesis (reviewed in Stafford, 1981; Hrazdina and Jensen, 1992). Integration of the resulting biochemical information with a wealth of accumulated genetic data led to a description of the sequence of genes involved in the production of anthocyanins in the maize aleurone in 1962 (Reddy and Coe, 1962). Enzymological information was also appearing during this time, with the first enzyme of the phenylpropanoid

pathway, phenylalanine ammonia-lyase (PAL), described in 1961, followed by cinnamate 4-hydroxylase (C4H) and the first flavonoid enzyme, chalcone isomerase (CHI) from soybean in 1967 (Koukol and Conn, 1961; Moustafa and Wong, 1967; Russell and Conn, 1967) (Figure 3.2). Eight years later, Kreuzaler and Hahlbrock (1975) (Figure 3.2) described the purification from parsley of a second enzyme, chalcone synthase (CHS), which functions at the entry point into the pathway. Soon thereafter, Styles and Ceska (1977) published a scheme for flavonoid biosynthesis that incorporated some 25 structural and regulatory genes functioning in diverse tissues of maize. This was followed by the isolation of numerous additional flavonoid enzymes and the corresponding genes (reviewed in Winkel-Shirley, 2001b), the first gene, for parsley CHS, being described in 1983 by Hahlbrock's group (Kreuzaler et al., 1983). During this period, Stafford raised the possibility that the enzymes of flavonoid metabolism, like those of the phenylpropanoid pathway, might be organized as one or more enzyme complexes (Stafford, 1974), and substantial experimental evidence in support of this idea was published in the mid-1980s by Hrazdina and his colleagues (Wagner and Hrazdina, 1984; Hrazdina and Wagner, 1985) (Figure 3.2).

Numerous recent reviews have summarized the extensive progress made in the flavonoid field since 1990, including advances in deciphering the signaling pathways that regulate expression of flavonoid genes as well as mechanisms controlling the intracellular distribution of flavonoid end products (Winkel-Shirley, 2001b; Vom Endt et al., 2002; Marles et al., 2003; Springob et al., 2003; Schijlen et al., 2004; Dixon et al., 2005). In addition, the first three-dimensional structures of several flavonoid enzymes were solved in the past few years, first for CHS and CHI from *Medicago truncatula*, solved by Noel's group (Ferrer et al., 1999; Jez et al., 2000), and then for anthocyanidin synthase [(ANS) also known as leucoanthocyanidin dioxygenase (LDOX)] from *Arabidopsis* by Wilmouth et al. (2002).

Still, major gaps remain in our understanding of flavonoid biochemistry, including the structural and biochemical basis of substrate- and stereospecificity for most flavonoid enzymes and the mechanisms by which flux is distributed among the various branch pathways. Unanswered questions also remain regarding the identity of several enzymes and precise sequence of events that lead to the major classes of flavonoid endproducts.

3. RECENT ADVANCES IN IDENTIFICATION AND CHARACTERIZATION OF THE PRIMARY FLAVONOID ENZYMES

3.1. Proanthocyanidin Biosynthesis

Despite extensive biochemical and genetic efforts over the past several decades to identify the enzymes that mediate flavonoid biosynthesis, a number of key steps have resisted elucidation. An important branch pathway that has long posed challenges in terms of defining the relevant biochemical steps is the one leading to the proanthocyanidins (a.k.a. condensed tannins) (reviewed in Dixon et al., 2005). This pathway is of substantial interest as a potential target for genetic modification to improve the yield, forage traits, and nutritional properties of crop species. A major recent breakthrough has been the isolation of genes encoding two key enzymes—anthocyanidin reductase (ANR) and leucoanthocyanidin reductase (LAR)—that provide the initiating and extension units for proanthocyanidin biosynthesis (Figure 3.1).

ANR was initially identified through characterization of the *banyuls* locus in *Arabidopsis* (Devic et al., 1999). The gene and enzyme now have been studied in detail in both *Arabidopsis* and *Medicago* (Xie et al., 2003, 2004a) and the enzyme activity has been described in *Camellia sinensis* and a number of other plant species (Punyasiri et al., 2004; Dixon et al., 2005). ANR, which is closely related in sequence to dihydroflavonol reductase (DFR) as well as the phenylpropanoid and isoflavonoid enzymes, cinnamoyl-CoA reductase, cinnamoyl alcohol dehydrogenase, and vestitone reductase, converts the product of ANS/LDOX, an anthocyanidin, to a 2,3-*cis*-2R,3R-flavan 3-ol (Figure 3.3). This discovery provided the first indication that ANS/LDOX has a role in both anthocyanin and proanthocyanidin biosynthesis and offered an explanation for the difference in stereochemistry between 2,3-*trans* isomers for flavonols and anthocyanins, and 2,3-*cis* isomers for the proanthocyanidin extension units in most plants. This is further supported by the isolation of two mutant alleles of *Arabidopsis* ANS/LDOX, *tt18* and *tds4*, in screens for plants deficient in proanthocyanidin biosynthesis, which also provided genetic evidence that ANS/LDOX precedes ANR in the pathway (Abrahams et al., 2003; Shikazono et al., 2003). A gene encoding another key enzyme of proanthocyanidin biosynthesis, LAR, was cloned from the legume *Desmodium uncinatum* and found to be a member of the plant reductase-epimerase-dehydrogenase (RED) superfamily, which also includes the gene for isoflavone reductase (Tanner et al., 2003). LAR generates (2,3-*trans*) catechin from leucoanthocyanidin, competing with ANS/LDOX for substrate to produce an alternative initiating unit for proanthocyanidin biosynthesis. Interestingly, LAR does not appear to exist in *Arabidopsis*, consistent with the presence of only 2,3-*cis* initiating and extension units in this species (Abrahams et al., 2003; Tanner et al., 2003). Although we have moved much closer to an understanding of this pathway

with the cloning and characterization of ANR and LAR, the mechanism by which the extension units are incorporated into proanthocyanidin polymers remains unknown (Dixon et al., 2005).

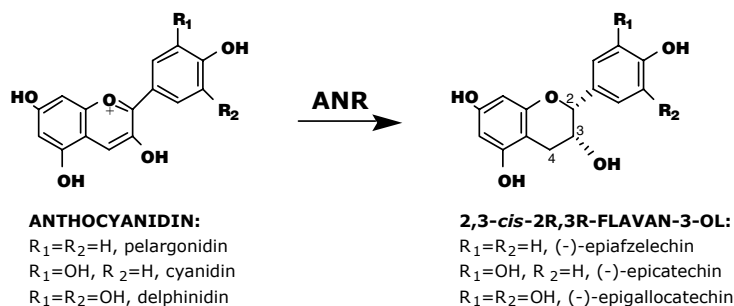


Figure 3.3 Reaction catalyzed by anthocyanidin reductase (ANR) (Xie et al., 2003).

3.2. Dihydroflavonol Reductase

In contrast to ANR, DFR has long been known to contribute to both anthocyanidin and proanthocyanidin biosynthesis (Heller et al., 1985; Reddy et al., 1987). Recently, Shimada et al. (2004) reported the characterization of DFR enzymes from *Spinachia oleracea* and *Phytolacca americana*. As with most other members of the order Caryophyllales, anthocyanins are entirely replaced in these plants by betalain pigments, which are derived from tyrosine via dihydroxyphenylalanine by a pathway unrelated to flavonoid metabolism (Strack et al., 2003). Recombinant DFR enzymes from these species are functional *in vitro*, producing cyanidin from dihydroquercetin; however, no such activity could be detected in extracts from seedlings or mature leaves of either species. This could be due to the low expression levels of the corresponding genes, which were assessed by Northern analysis and semiquantitative RT-PCR in various tissues. It also was suggested that these enzymes participate in proanthocyanidin biosynthesis, although these compounds were not present in the vegetative tissues examined in this study. Another possibility is that the DFR enzymes in these plants have lost the ability to interact with other components of a flavonoid enzyme complex, so that only flavonols are synthesized in tissues where enzymes of the proanthocyanidin pathway, such as ANS/LDOX, ANR, or LAR, are not present. More work is needed on this interesting system to assess the specific role(s) of the DFR enzymes in these species and how the flavonoid and betalain pathways have evolved in the Caryophyllales.

3.3. The 2-ODD Enzymes

Advances in recombinant protein expression technology and substrate/product detection methods have fueled rapid advances in the biochemical characterization of

numerous key flavonoid enzymes in recent years. This is particularly true of members of the 2-ODD enzyme family for which conventional biochemical analysis of plant extracts had been limited by the oxygen sensitivity of the enzyme and its susceptibility to cleavage by endogenous enzymes (Britsch and Grisebach, 1986; Lukacin et al., 2000). Four 2-ODDs long have been known to function in flavonoid metabolism in all plants: flavanone 3-hydroxylase (F3H), flavonol synthase (FLS), flavone synthase (FNSI), and ANS (Figure 3.1). A fifth 2-ODD, flavonol 6-hydroxylase (F6H), was more recently identified in the semiaquatic weed, *Chrysosplenium americanum*, and shown to catalyze hydroxylation prior to O-methylation at the 6 position of the A ring, a novel activity for this class of enzymes (Anzellotti and Ibrahim, 2000, 2004) (Figure 3.4).

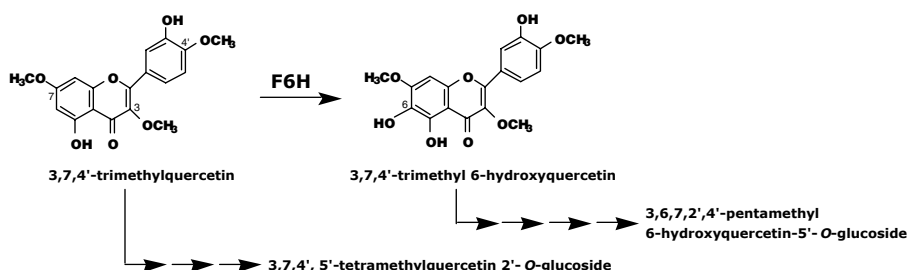


Figure 3.4 The reaction catalyzed by the 2-ODD enzyme, flavonol 6-hydroxylase (F6H) from *Chrysosplenium americanum* (Anzellotti and Ibrahim, 2000).

A great deal of biochemical information on the flavonoid 2-ODD enzymes has emerged since the mid-1980s, starting with the seminal work of Lothar Britsch and colleagues (reviewed in Prescott and John, 1996). Recent studies incorporating HPLC analysis into enzyme assays using conventional radiolabeled substrates, or increasingly unlabeled substrates, have provided evidence for overlapping substrate and catalytic specificities among these enzymes. The first indications for this overlap came from studies on FLS enzymes from *Arabidopsis* and *Citrus unshiu*, which were found to exhibit substantial F3H activity in addition to their primary enzymatic activity in the terminal step of flavonol production (Prescott et al., 2002; Lukacin et al., 2003). In addition, *in vitro* experiments with recombinant ANS suggested that the enzyme could produce not only its natural product, cyanidin, but depending on the C-4 stereochemistry of the leucocyanidin substrate possibly also dihydroquercetin and quercetin (Turnbull et al., 2003). Turnbull et al. (2004) subsequently compared the substrate preferences and stereospecificities of F3H, FLS, and ANS enzymes from *Arabidopsis* and *Petunia hybrida*. The results of this study indicated that the reactions catalyzed by the four enzymes all proceed via an initial oxidation at the C-3 position. However, it also grouped F3H/FNSI and FLS/ANS into two distinct subfamilies. The former have a very narrow substrate preference, recognize only a single stereoisomer of their natural substrate, naringenin, and are classified as C-3 β -face oxygenases. In contrast, FLS and ANS

are C-3 α -face oxygenases that efficiently recognize different stereoisomers of their natural, as well as several unnatural, substrates. The flavonoid 2-ODDs clearly offer an excellent experimental system for defining the molecular determinants of the substrate and stereoselectivities of this important class of enzymes. Domain swapping experiments between *Petunia* F3H and *Citrus* FLS already have provided evidence that the selectivity of F3H is not conferred by the 52 C-terminal amino acids (Wellmann et al., 2004). Further enhancement of methods for biochemical characterization of 2-ODDs, such as a recently described fluorescence-based assay (McNeill et al., 2004), should help facilitate these types of studies in the future. Enormous promise also lies in combining biochemical analyses with structural approaches to understanding 2-ODD enzyme function. A high-resolution crystal structure for *Arabidopsis* ANS, including one for the enzyme in complex with enantiomerically pure dihydroquercetin, already is providing new insights into the mechanism of stereoselective C-3 hydroxylation by this enzyme that are likely also to apply to other members of this class of enzymes (Wilmouth et al., 2002).

3.4. Common Activities Specified by Members of Different Enzyme Classes

There are now three different examples of the overlapping contributions of 2-ODDs and cytochrome P450s in the evolution of flavonoid metabolism. Work by Shin-ichi Ayabe's group (Sawada et al., 2002; Sawada and Ayabe, 2005) involving site-directed mutagenesis of the P450 enzyme, isoflavone synthase (IFS), suggests that this enzyme might have been derived from an ancestral CYP93 enzyme that also gave rise to flavanone 2-hydroxylase and flavone synthase II (FNSII) (Figure 3.1). These experiments indicate that IFS could have evolved via an intermediate enzyme having flavanone 3-hydroxylase activity. Interestingly, FNS activity is specified by either a P450 (FNSII) or a 2-ODD (FNSI) enzyme in different plant species (Britsch, 1990; Martens and Forkmann, 1999). It is conceivable that F3H was represented by members of these two different enzyme classes in ancestral plant species. There is now also evidence that F6H activity, first described as a 2-ODD enzyme in *Chrysosplenium americanum* (Anzellotti and Ibrahim, 2000, 2004), is specified by a P450 monooxygenase in *Tagetes* species (Halbwirth et al., 2004). The activities differ in that the enzyme from *Chrysosplenium americanum* accepts methylated flavonols, while the *Tagetes* enzyme does not. These findings underscore the remarkable evolutionary plasticity of plant secondary metabolism and the potential to achieve similar ends through entirely unrelated means. It could be interesting to consider whether similar evolutionary processes may have occurred in other metabolic systems. Further evidence for the biochemical plasticity of the flavonoid pathway comes from analyses of the substrate and product specificities of FLS and DFR. These enzymes carry out unrelated reactions in flavonoid biosynthesis (Figure 3.1) and belong to entirely different enzyme classes: the 2-ODD and SDR superfamilies. However, both enzymes utilize primarily dihydroflavonols, but also have been shown to convert flavanones (e.g., naringenin and eriodictyol) to dihydroflavonols in the case of *Arabidopsis* and *Citrus* FLS, an activity traditionally

associated with F3H (Prescott et al., 2002; Lukacin et al., 2003), or to flavan 4-ols in the case of DFR from *Dahlia variabilis*, *Gerbera hybrida*, *Zea mays*, and *Medicago*, an activity previously named flavanone 4-reductase (FNR) (Fischer et al., 1988, 2003; Halbwirth et al., 2003; Xie et al., 2004b) (Figure 3.5).

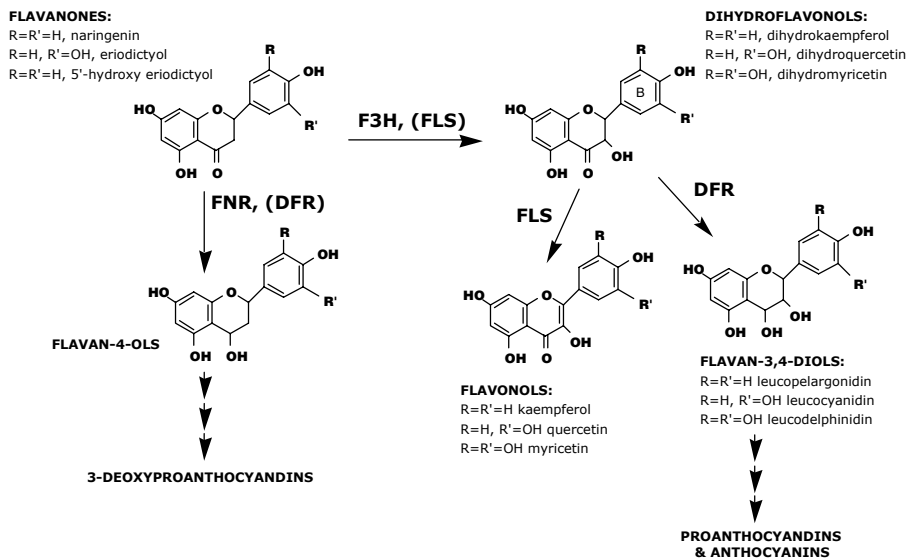


Figure 3.5 Flexible substrate recognition of flavonol synthase (FLS) and dihydroflavonol 4-reductase (DFR) (Prescott et al., 2002; Lukacin et al., 2003; Xie et al., 2004b).
 Abbreviations: F3H, flavanone 3-hydroxylase; FNR, flavanone 4-reductase.

The broad substrate specificity of DFR has led to the suggestion that it is the level of F3H activity that ultimately determines the type of anthocyanin that accumulates in maize silks (Halbwirth et al., 2003). Although FLS and DFR do recognize a similar set of substrates, a difference between these enzymes is that only DFR exhibits a strong stereospecificity. For example, two recently characterized DFR enzymes from *Medicago* convert only the 2R,3R form of dihydroquercetin (Xie et al., 2004b), consistent with the stereochemistry of naturally occurring flavonols and anthocyanins. These enzymes therefore are likely to provide a useful experimental system for understanding the determinants of substrate and stereospecificity in individual enzymes.

Efforts also continue to be focused on elucidating how the preference of DFR for its natural dihydroflavonol substrates is determined (Punyasiri et al., 2004; Xie et al., 2004b). DFR enzymes from different plant species vary in acceptance of dihydroflavonols with different B-ring oxidation states (Figure 3.5), a characteristic that has been of long-standing interest with regard to engineering flower color (Meyer et al., 1987). There long has been evidence for the role of a 26 amino acid region, and more recently for one residue in particular, in determining DFR substrate

specificity (Beld et al., 1989; Johnson et al., 2001). However, the recent analysis of the *Medicago* enzymes shows that the molecular basis of this specificity, including the roles of other active site residues, remains to be elucidated (Xie et al., 2004b). Accumulating information on members of the SDR class of enzymes in a wide range of species, including more than 40 three-dimensional structures now deposited in the protein database, suggests that new insights will soon be forthcoming (Oppermann et al., 2003).

4. ADVANCES IN ELUCIDATING FLAVONOID MODIFICATION REACTIONS

In addition to the ongoing interest in the primary enzymes of flavonoid metabolism, there has been a growing focus on enzymes that perform the substitution reactions responsible for the tremendous structural and functional diversity of flavonoids found in nature. This is driven in part by opportunities for metabolic engineering of flavonoid biosynthesis for agronomic and nutritional improvement of plants. Methylases, acetyltransferases, and GTs confer many of the ultimate chemical and bioactive properties of flavonoids, for example, modulating flower color or enhancing the activity of these compounds for use in defense against herbivores and pathogens. These modifications also may play an important part in controlling the distribution of flux across branch pathways by altering intermediates to favor utilization by one or more competing enzymes at critical branch points. It is noteworthy that many of the substitution reactions are quite species-specific and most are still poorly understood both in terms of enzymology and their biochemical or physiological significance.

4.1. Flavonoid Methyltransferases

A variety of *O*-methylated flavonoids have been described, involving substitutions at the 3, 5, 6, 7, 4' or 5' positions (for example, Figure 3.4). Huang et al. (2004) have recently described the purification and biochemical characterization of a 3-*O*-methyltransferase from *Serratula tinctoria* that exhibits a preference for quercetin over other flavonols. This enzyme is of particular interest because 3-*O*-methylation confers a number of distinct activities to quercetin, including antiinflammatory and antiviral properties. Thus, the enzyme clearly has potential metabolic engineering applications and cloning of the corresponding gene is underway. Other studies have focused on how the stereo- and regiospecific addition of sugars and other groups is controlled. Comparison of the substrate preferences and kinetic characteristics of variants of a class I OMT from the ice plant, *Mesembryanthemum crystallinum*, indicates that the N-terminus of this enzyme is an important determinant of these properties (Vogt, 2004). Deletion of 11 N-terminal residues allows the enzyme to add sugars to not only the 3' and 6 positions observed for the native plant protein, but also to the 5 position (Figure 3.6). However, it also reduces the overall catalytic efficiency of the enzyme, apparently affecting turnover rate rather than substrate

binding affinity. Efforts are underway to solve the crystal structure of this enzyme in order to understand these observations at a molecular level. Interestingly, characterization of a novel 4'-O-methyltransferase from *Catharanthus roseus* indicates that, at least for the four major 2-ODDs that function in flavonoid synthesis, methylation at the 4' position did not reduce substrate utilization (Schröder et al., 2004). This suggests that, although this modification may alter the biological activity of flavonoid end products, it does not by itself control flux into different branch pathways.

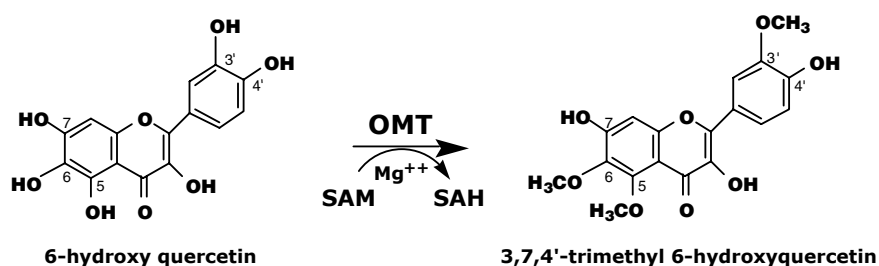


Figure 3.6 Regiospecific methylation of 6-hydroxy quercetin catalyzed by a phenylpropanoid and flavonoid O-methyltransferase from the ice plant, *Mesembryanthemum crystallinum* (Vogt, 2004).

4.2. Flavonoid Glycosyltransferases

The addition of sugar groups has been well documented to enhance the solubility of flavonoids, as well as many other metabolites, and is likely to be critical for the transport and storage of these compounds at the final destinations in the vacuole or cell wall.

Most flavonoid end products exhibit complex glycosylation patterns involving the addition of one or more glucose, rhamnose, or other sugars. Recent progress in understanding how this occurs includes elucidation of the terminal steps in the biosynthesis of maysin, a flavone produced in maize that provides resistance against the corn earworm, *Helicoverpa zea*. Quantitative trait locus and metabolite analysis of the maize *salmon silk* (sm) phenotype by McMullen and colleagues (2004) has shown that the *sm1* and *sm2* genes encode or control a glucose modification enzyme and a rhamnosyl-transferase activity, respectively, in the maysin pathway. This analysis has made possible the ordering of the final intermediates in the pathway from the flavone, luteolin, to isoorientin, to rhamnosylisoorientin, and then to the

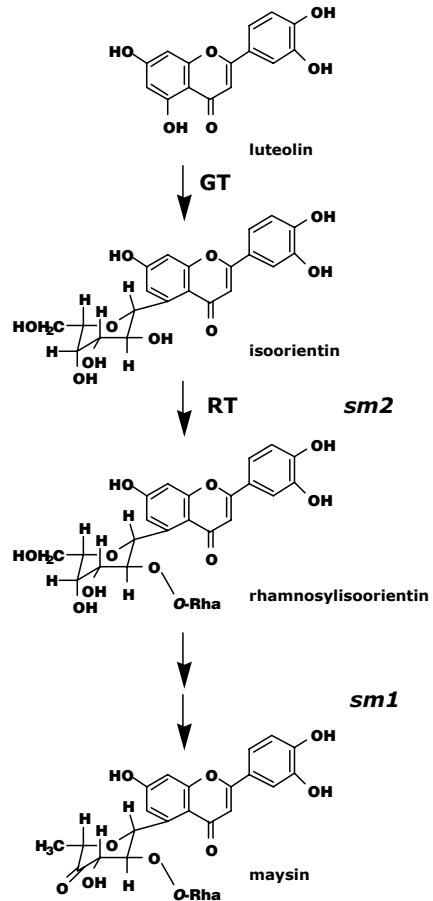


Figure 3.7 Pathway for the synthesis of maysin from the flavone luteolin. The *sm1* and *sm2* loci are now known to encode or control the terminal steps (Vogt, 2004).
Abbreviations: GT, glucosyl transferase; RT, rhamnosyl transferase.

bioactive product, maysin (Figure 3.7). In *Citrus*, a gene encoding a 1,2 rhamnosyltransferase, a key determinant of bitterness in fruits such as pummelo and grapefruit, has been described (Frydman et al., 2004). The enzyme is more closely related to the only other cloned flavonoid-glucoside rhamnosyl transferase, an enzyme from *Petunia*, than other GTs, including enzymes that conjugate rhamnose directly to the flavonoid backbone. This is somewhat surprising as the *Petunia* adds the sugar to a 3-*O*-glucoside substrate at the 6 position of the glucose moiety, while the *Citrus* enzyme is specific for 7-*O*-glucoside substrates and places rhamnose at the 2 position (Figure 3.8). Because the RT that participates in maysin biosynthesis also adds rhamnose at the 2 position of glucose, but onto a 6-*O*-glucoside substrate, it will be interesting to compare the substrate binding and reaction mechanisms for these three enzymes. Progress also has been made in the identification of genes encoding UDP-rhamnose:flavonol-3-*O*-rhamnosyltransferase and UDP-glucose:flavonol-3-*O*-glycoside-7-*O*-glucosyltransferase in *Arabidopsis* based on homology to other known flavonoid GTs and combined genetic and biochemical analyses (Jones et al., 2003). The presence of these activities in *Arabidopsis* is consistent with the structures of the flavonol glycosides of this species (Veit and Pauli, 1999). Using a more global approach to developing new tools for engineering the production of small molecule glycosides, Dianna Bowles's group has surveyed 91 recombinant GTs from *Arabidopsis*, 29 of which were found to be capable of glycosylating quercetin, reflecting the substrate promiscuity of these enzymes (Lim et al., 2004). These GTs should be extremely useful for efforts to define the determinants of GT substrate specificity and also may have substantial practical value for directing the *in vitro* synthesis of commercially important mono- and diglucosides. It also has recently been reported that *dusky* mutants of the Japanese morning glory, which have reddish-brown or purple-gray flowers rather than bright red or blue flowers, are deficient in a novel anthocyanin glucosyltransferase (Morita et al., 2005).

As with many other enzymes, the molecular basis of the substrate and regioselectivity of the flavonoid GTs is of significant interest. A single amino acid change recently has been shown to increase the sugar donor specificity of a UDP-galactose:anthocyanin galactosyltransferase from *Aralia cordata* to include UDP-glucose (Kubo et al., 2004). However, UDP-galactose specificity did not appear to be associated with the same residue, indicating that much remains to be learned about the catalytic mechanism of this group of enzymes. Cloning and characterization of a novel glucuronyltransferase (UGAT) from red daisy (*Bellis perennis*) that is believed to confer solubility and stability to the pigment in flowers suggests that this enzyme has a relatively broad substrate specificity (Sawada et al., 2005). This may allow the enzyme to participate in a metabolic grid at the end of pigment biosynthesis in which either malonylation at the 6 position or glucuronosylation at the 2 position of glucose may take place first, similar to what has been proposed for the late steps in pigment biosynthesis in red *Perilla* leaves (Yamazaki et al., 1999). Together, these findings are opening new doors in understanding substrate binding and regiospecificity of the various modification

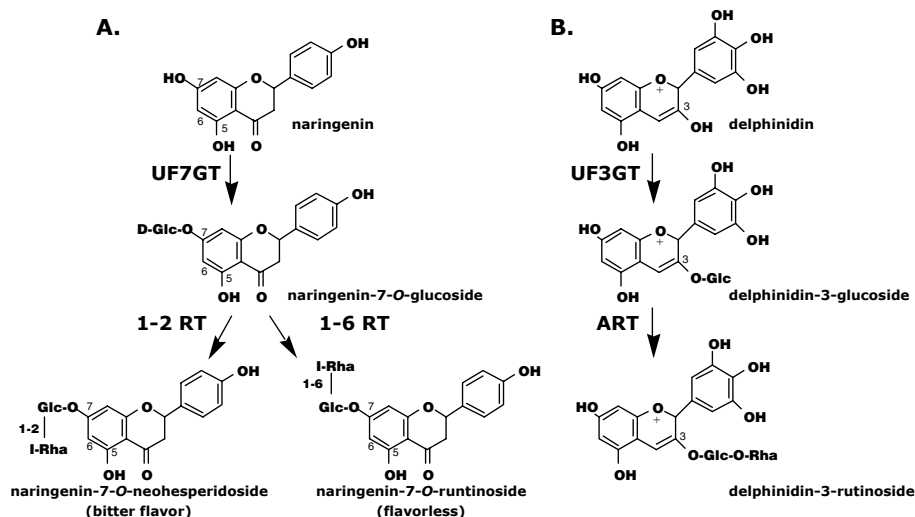


Figure 3.8 Glycosylation of the flavanone, naringenin, in Citrus (Frydman et al., 2004) and the anthocyanidin, delphinidin, in Petunia (Brugliera et al., 1994). Abbreviations: UDPG flavonoid 3 or 7-glucosyltransferase (UF3GT or UF7GT); anthocyanin rhamnosyltransferase (RT).

enzymes that function in secondary pathways, information that is clearly important for efforts to engineer the chemical and biological characteristics of flavonoids and other metabolites.

4.3. Flavonoid Acetyltransferases

Although acetylation of flavonoids has been known for some time, it is less common and not as well understood as the other modifications. It has been suggested that acyl groups are effective competitors for addition of water at the 2 and 4 position and may serve to stabilize the anthocyanin chromophore (Stafford, 1990). Insights on substrate selection of flavonoid acetylases recently has come from isolation and characterization of a novel anthocyanin malonyltransferase from scarlet sage (*Salvia splendens*) (Suzuki et al., 2004). This enzyme catalyzes the second malonylation reaction of anthocyanins in this species, adding malonyl at the 4 position of the 5-glucosyl moiety to produce salvinin flower pigments (Figure 3.9). Sequence comparison with the enzyme that catalyzes the previous step, malonylation at the 6 position of this glucose moiety, indicates that even these two enzymes have very

similar substrate specificities; they evolved from different branches of the BAHD acyltransferase family.

4.4. Peroxidases

In addition to the modifications that occur in the cytoplasm, additional processing can occur at the final destination in the cell wall or the vacuole. In particular, anthocyanins can be oxidized by peroxidases to become brown or colorless. Information on flavonoid-specific peroxidases is just beginning to emerge, even though correlations between peroxidase activity and flower color were made more than 90 years ago by Keeble and Armstrong (1912a, 1912b). Wang et al. (2004) recently have described the cloning and characterization of a peroxidase from *Raphanus sativus* (Chinese red radish) that they propose to have a function in or at the vacuole in the oxidation of anthocyanins. This enzyme appears to be quite common, at least among the *Brassicaceae*, grouping phylogenetically with six of seven peroxidases identified in horseradish, and 7 of 73 putative peroxidases from *Arabidopsis*.

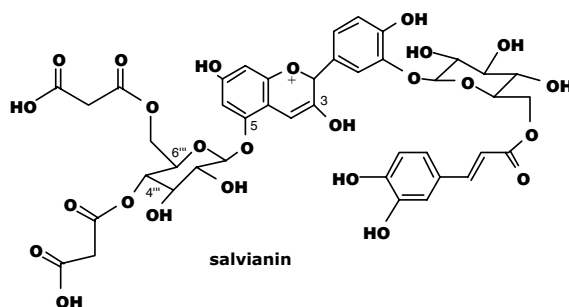


Figure 3.9 Structure of the anthocyanin pigment, salviainin, from *Salvia splendens*. The terminal steps in the synthesis of this compound involve addition of malonyl to the 5-glycosyl moiety, first at the 6 and then at the 4 position (Suzuki et al., 2004).

5. ENGINEERING OF FLAVONOID METABOLISM

The wealth of information that has resulted from decades of biochemical and molecular genetic characterization of flavonoid metabolism increasingly seems to underscore just how much remains to be learned about this system. Efforts to engineer flavonoid metabolism is one of the best illustrations of this, with early work resulting in some striking success stories, such as the use of a maize *DFR* gene to produce a new flower color in *Petunia* (Meyer et al., 1987). In other well-known cases, introduction of flavonoid transgenes has resulted in unstable phenotypes and in fact provided one of the first examples of cosuppression (Jorgensen, 1995). Gene downregulation using RNAi technology at least now appears to provide a way to

target genes in a highly specific manner, as illustrated in modulation of flower color in *Torenia hybrida* using RNAi against CHS (Fukusaki et al., 2004).

At the same time, the unexpected outcomes of attempts to engineer flavonoid biosynthesis continue to generate new insights into how metabolism really operates in cells. For example, two recent engineering efforts have uncovered new clues as to how flux through competing branch pathways is controlled. Dixon's laboratory has shown that the bottleneck for engineering isoflavonoid synthesis in *Arabidopsis* involves competition for flavanone between the introduced enzyme, isoflavone synthase (IFS), and the endogenous FLS1 enzyme (Figure 3.1) (Liu et al., 2002). Similarly, anthocyanin production in a white-flowered *Petunia* line has been achieved, not just by enhancing DFR expression, but also by downregulating expression of FLS; the highest levels of anthocyanin production were achieved by using both approaches together (Davies et al., 2003). These findings are building a case for the FLS reaction as a key point in the regulation of flux into the branch pathways of flavonoid metabolism. There also are examples of the effects of engineering competition with the entry point enzyme, CHS, including reduced coloration of *Petunia* and *Peuraria montana* flowers upon introduction of chalcone reductase, which in legumes shifts flux into the isoflavonoid pathway (Davies et al., 1998; Joung et al., 2003). Similarly, reduced flower color and fertility were reported in tobacco upon introduction of a stilbene synthase gene, which normally sends flux into the stilbene branch pathway (Fischer et al., 1997). Some of these negative effects can be ameliorated by selecting lines with altered expression of the endogenous pathway or with moderate levels of transgene expression (for example, Fettig and Hess, 1999). All these observations indicate that a great deal remains to be learned about the mechanisms normally used by cells to distribute flux among competing branch pathways.

Reconstitution in yeast of parts of the flavonoid and isoflavonoid pathways, as well as early steps in phenylpropanoid metabolism, is another application of metabolic engineering that may provide insights into the cellular organization and regulation of these enzyme systems. Yu's group has demonstrated the feasibility of expressing CHI, F3H, and FNSII in yeast cells to produce both the natural products from supplied chalcone precursors as well as novel compounds when uncommon substrates were provided (Ralston et al., 2005). This system is proposed to offer a unique platform for functional analysis of these enzymes, including their physical and genetic interactions. However, a similar study of the first enzymes of general phenylpropanoid metabolism from poplar (two isoforms of PAL, cinnamate 4-hydroxylase, and a cytochrome P450 reductase), failed to detect channeling of *trans*-cinnamic acid, even though this has been demonstrated for the corresponding enzymes in *Nicotiana tabacum* (tobacco) cells (Rasmussen and Dixon, 1999). Individual isoforms of tobacco PAL behave very differently in terms of association with the ER and presumably other phenylpropanoid enzymes (Achnine et al., 2004), which suggests that it could be the isoform used in these experiments rather than the yeast that is responsible for the lack of corroboration of the results obtained in tobacco.

6. FRONTIERS IN THE STUDY OF FLAVONOID METABOLISM

The localization of flavonoid metabolism and how this impacts the deposition of end products is another area with a long history of study where new information and new questions continue to arise. Our laboratory recently has demonstrated the presence of CHS and CHI in the nucleus of *Arabidopsis* cells (Saslowsky et al., 2005). This finding is consistent with several reports from other groups of the presence of flavonoids in nuclei in such diverse species as *Arabidopsis*, *Brassica napus*, *Flaveria chloraefolia*, *Picea abies*, *Tsuga Canadensis*, and *Taxus baccata* (Grandmaison and Ibrahim, 1996; Hutzler et al., 1998; Kuras et al., 1999; Peer et al., 2001; Buer and Muday, 2004; Feucht et al., 2004). These studies have raised interesting questions regarding the physiological roles of flavonoids, speculated to include protecting DNA from UV and oxidative damage (Feucht et al., 2004) or controlling the transcription of genes required for growth and development such as auxin transport proteins (Grandmaison and Ibrahim, 1996; Kuras et al., 1999; Buer and Muday, 2004). However, it also begs the question of how intracellular distribution of the enzymes is controlled. There is good evidence that flavonoid synthesis in the cytoplasm proceeds via an enzyme complex, tethered to the endoplasmic reticulum by the cytochrome P450 enzyme, flavonoid 3'-hydroxylase. However, this enzyme cannot be the assembly point for an enzyme complex in the nucleus; also, only CHI is sufficiently small enough to enter the nucleus on its own, but only CHS contains a predicted nuclear localization signal (NLS). Thus, either the other enzymes required to synthesize flavonoids such as quercetin 3-sulfate in *Flaveria chloraefolia* (Grandmaison and Ibrahim, 1996) are cotransported with CHS, or NLSs are formed upon association of these enzymes. At present the mechanisms by which cells regulate the distribution of flavonoid metabolism to these different compartments remains entirely unknown.

As already alluded to, one of the most important recent developments in the effort to understand the organization and operation of flavonoid metabolism has been the application of the tools of structural biology. The release over the past 5 years of the three-dimensional structures of CHS and CHI from *Medicago truncatula* and for ANS for *Arabidopsis* has enhanced dramatically our understanding of several of the key reactions of flavonoid metabolism (Jez et al., 2000, 2002; Wilmouth et al., 2002; Austin and Noel, 2003). Homology-based modeling is proving useful also in the analysis of numerous other enzymes where structures are available for even distantly related enzymes, including the 2-ODD (F3H, FLS, FNSI, and F6H), cytochrome P450 (FNSII, IFS, and flavonoid 3'-hydroxylase, F3'H), and SDR (DFR and ANR) enzymes that constitute the flavonoid pathway. This holds true for several modifying enzymes, with substantial structural information already available for OMTs (*O*-methyltransferases) and evidence that the Family I class of GT enzymes will be similar in structure to the GT-B enzymes, for which several crystal structures have been solved (Lim et al., 2004). In addition to the examples cited above, Yang et al. (2004) have described the use of docking simulations to model substrate in the active site of a flavonoid OMT from *Arabidopsis* in order to develop a mechanistic explanation for differences between the substrate preferences of this enzyme and that of caffeic acid OMT, the enzyme

used as the template for homology modeling. Molecular modeling also is being used as part of a functional genomics study of the *Arabidopsis* cytochrome P450 enzymes by Schuler's laboratory. Initial efforts have focused on four enzymes of phenylpropanoid metabolism, including F3'H (Figure 3.10), the structures of which were modeled on the crystal structures of four bacterial and one mammalian P450 enzyme (Rupasinghe et al., 2003). Analysis of these models indicated that, despite as little as 13% sequence identity among these proteins, the structural cores and several loop regions are highly conserved. Substrate docking simulations suggested that the enzymes use a similar substrate recognition mechanism and are informing mutagenesis experiments aimed at clarifying the roles of specific residues in defining the substrate preferences of these enzymes.

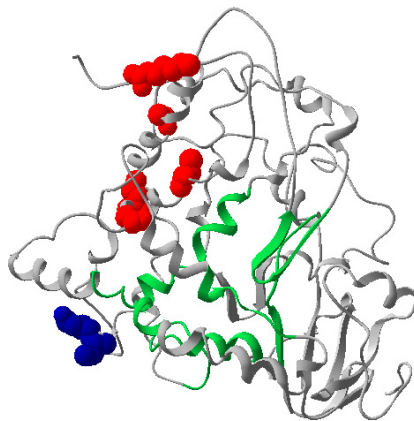


Figure 3.10 Homology model of *Arabidopsis* F3'H developed by the Schuler laboratory (Rupasinghe et al., 2003). The structure was visualized using Swiss-PdbViewer 3.7. Side chains of residues corresponding to the P450 signature motif, which includes the heme-binding cysteine (red), and the DT pair that mediates dioxygen activation (blue) are shown. The six regions that determine substrate specificity (SRS1-6) are highlighted in green. See Color Section for figure in colors.

The availability of authentic structures and homology models already has enhanced radically our understanding of reaction mechanisms. It will be interesting to see how these types of modeling experiments perform overall as predictive tools for enzyme function. The promise of this approach is underscored by the fact that the substrate and product specificity of a *Gerbera* CHS-like protein were accurately predicted by modeling the active site architecture based on the *Medicago* CHS crystal structure (Ferrer et al., 1999). Structural information also will be essential for understanding the organization and intracellular distribution of the flavonoid pathway. For example, our laboratory has begun fitting docking simulations of homology models of *Arabidopsis* CHS and CHI to data generated by small-angle neutron-scattering experiments to explore the structural basis of the interaction of

these two enzymes (Dana, Martins, Kreuger, and Winkel, unpublished data). The fact that many other metabolic systems are composed of members of the same classes of enzymes suggests that what is learned from this highly tractable experimental system will have broad applicability in understanding the organization of cellular metabolism.

7. CONCLUSIONS

Studies of flavonoid metabolism are increasingly intertwined with efforts to understand a wide array of other primary and secondary metabolic systems. The growing accessibility of sophisticated tools for determining the biochemical and structural characteristics of enzymes is driving this work to new levels, even as it adds to our appreciation for the true complexity of cellular metabolism. The practical implications of new knowledge for engineering enhanced agronomic and nutritional traits in plants also are more and more evident. It seems likely that flavonoid metabolism will continue to serve as an important and tractable experimental model for efforts to understand cellular metabolism for some time to come.

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