Evolution of flavone synthase I from parsley flavanone 3β-hydroxylase by site-directed mutagenesis

Corresponding author:
Stefan Martens, Institut für Pharmazeutische Biologie, Philipps-Universität Marburg, Deutschhausstr. 17A, 35037 Marburg, Germany.
Tel.: 0049-6421-2822416,
Fax: 0049-6421-2825366
Email: stefan.martens@staff.uni-marburg.de

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Evolution of flavone synthase I from parsley flavanone 3β-hydroxylase by site-directed mutagenesis

Yvonne Helen Gebhardt¹, Simone Witte¹, Holger Steuber², Ulrich Matern¹ and Stefan Martens¹

¹ Institut für Pharmazeutische Biologie, Philipps-Universität Marburg, Deutschhausstr. 17A, 35037 Marburg, Germany
² Institut für Pharmazeutische Chemie, Philipps-Universität Marburg, Marbacher Weg 6, 35037 Marburg, Germany
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Corresponding author: Stefan Martens, Fax: 0049-6421-2825366, Email: stefan.martens@staff.uni-marburg.de
Abstract

Flavanone 3β-hydroxylase (FHT) and flavone synthase I (FNS I) are 2-oxoglutarate-dependent dioxygenases with 80% sequence identity which catalyze distinct reactions in flavonoid biosynthesis. However, FNS I has been reported exclusively from few Apiaceae species while FHTs are more abundant. Domain swapping experiments joining the N-terminus of parsley FHT with the C-terminus of parsley FNS I and vice versa revealed that the C-terminal portion is not essential for FNS I activity. Sequence alignments identified 26 amino acid substitutions conserved in FHT vs. FNS I genes. Homology modelling based on the related anthocyanidin synthase structure assigned seven of these amino acids (FHT/FNS I: M106T, I115T, V116I, I131F, D195E, V200I, L215V and K216R) to the active site. Accordingly, FHT was modified by site-directed mutagenesis creating mutants encoding from one to seven substitutions, which were expressed in yeast for FNS I and FHT assays. The exchange I131F in combination with either M106T and D195E or L215V and K216R replacements was sufficient to confer some FNS I side activity. Introduction of all seven FNS I substitutions into the FHT sequence, however, caused a nearly complete change in enzyme activity from FHT to FNS I. Both FHT and FNS I were proposed to initially withdraw the ‘β-face’-configurated hydrogen from carbon-3 of the naringenin substrate. Our results suggest that the 7-fold substitution affects the orientation of the substrate in the active site pocket such that this is followed by syn-elimination of hydrogen from carbon-2 (FNS I reaction) rather than the rebound hydroxylation of carbon-3 (FHT reaction).
Introduction

Flavones and flavonols are the predominant flavonoids found in tissues of the *Apiaceae* species (Harborne, 1971; Harborne and Williams, 1972; Harborne and Baxter, 1999). Significant functions were ascribed to these metabolites for the growth and propagation of the plants as well as for the adaptation to ecological niches. Flavonoids have been shown to protect from ultraviolet radiation, provide pigmentation, mediate the plants’ interaction with insects or microbes, and act as feeding deterrents and phytoalexins (Harborne and Williams, 2000; Martens and Mithöfer, 2005). Flavones, *i.e.* apigenin, are formed by direct 2,3-desaturation of natural flavanones such as (2S)-naringenin (Fig. 1). In Apiaceae, this reaction is catalyzed by a soluble Fe$^{2+}$/2-oxoglutarate-dependent dioxygenase (2-ODD), flavone synthase (FNS I), whereas FNS II, a cytochrome P450-dependent monooxygenase, was found in all other flavone-producing plants investigated so far. Flavonols are formed from flavanones by sequential hydroxylation of carbon-3 and 2,3-dehydration involving flavanone 3β-hydroxylase (FHT) and flavonol synthase (FLS) (Fig. 1), although *in vitro* FLS has the capability of catalyzing both steps (Martens et al., 2003). FHT and FNS I or II thus compete for flavanones as common substrates, and the product of FHT may be delivered to the anthocyanidin branch pathway instead of desaturation by FLS (Fig. 1). FHT also belongs to the superfamily of 2-ODDs and is closely related to FNS I. Both enzymes had been proposed to attack the flavanone substrate in an identical fashion and withdraw initially the β-configurated hydrogen (*trans* to B-ring substitution) from carbon 3 (Fig. 1).

Considerable research has been dedicated to the mechanism of 2-ODD catalysis because of the relevance of these enzymes in the metabolism of microorganisms (antibiotics), plants (hormones, pigments) or mammals (connective tissue diseases, hypoxia-inducible factor). Two electrons are gained from the decarboxylation of 2-oxoglutarate and transferred to the Fe(II) in the enzyme active center, forming a highly reactive ferryl intermediate which mobilizes molecular oxygen for hydroxylation, desaturation, epoxidation, ring closure or expansion reactions (Prescott and Lloyd, 2000). Members of the 2-ODD superfamily do not always show close sequence identities but rather appear to cluster in one of three groups of related enzymes or fall into a fourth group of unrelated sequences (Hogan et al., 2000; Prescott and Lloyd, 2000). FHT and FNS I were assigned to group I of this superfamily together with, for example, microbial isopenicillin N synthase (IPNS) and deacetoxycephalosporin C synthase (DAOCS) having in common a HXDX$_{55}$HX$_{10}$RXS motif (Borovok et al., 1996; Prescott and Lloyd, 2000). These residues are particularly important for
enzyme activity as revealed by site-directed mutagenesis (Lukacin and Britsch, 1997; Myllyharju and Kivirikko, 1997). Furthermore, IPNS (Roach et al., 1995; Roach et al., 1997) and DAOCS (Roach et al., 1995; Roach et al., 1997; Vålegard et al., 1998; Lloyd et al., 1999; Wilmouth et al., 2002), as well as anthocyanidin synthase (ANS) from Arabidopsis thaliana (Wilmouth et al., 2002) as another group I dioxygenase, were crystallized and revealed that these residues comprise the metallocenter and 2-oxoglutarate binding site. For a detailed review on 2-ODDs see (Prescott and Lloyd, 2000; Clifton et al., 2006).

The evolution of FNS I in species of the Apiaceae ascribes an essential role to flavones for the plants’ existence and propagation. Moreover, the confinement of FNS I to one evolutionary advanced plant family as compared to the more abundant expression of FHTs suggested that FNS I developed much later than FHT. The close relationship of the FNS I polypeptide with those of FHTs and alignments with other 2-ODD sequences cloned from Apiaceae thus led to the hypothesis of gene duplication and subsequent change of function (Gebhardt et al., 2005). Very few conserved differences became apparent on alignment of FNS I and FHT sequences from parsley, which were likely to determine the divergent catalytic activities. The Apiaceae presumably benefit from stable maintenance of the new FNS I gene, which leads to the accumulation of flavones, and the selective advantage has precluded any further diversification of 2-ODD functionality towards the destructive turnover of flavones. However, experimental evidence for this hypothesis has been lacking. In order to identify the amino acid residues essential for FNS I activity, we constructed chimera from fully functional parsley FHT and parsley FNS I (Martens et al., 2003) and generated step-by-step mutants of the FHT. The chimera functionally expressed in yeast revealed the primary importance of the N-terminal enzyme portion for FNS I catalysis, and site-directed mutagenesis identified the minimal requirement of three amino acid substitutions to shift the FHT towards FNS I activity.
Results

Cloning of 2-ODDs from Apiaceae and sequence alignment

In addition to the FHT and FNS I sequences already identified from various species of the Apiaceae (Gebhardt et al. 2005), full length FNS I from Aethusa cynapium (DQ683350), Angelica archangelica (DQ683352) and Cuminum cyminum (DQ683349) as well as FHT from Aethusa cynapium (DQ683351) were cloned by PCR amplification and verified by functional expression. Alignments of all these translated polypeptides corroborated the previous finding of 27 amino acids differently conserved in FNS I vs. FHT, albeit Angelica archangelica FNS I is exceptional with Val312 instead of the otherwise common Ile (Fig. 2). Nevertheless, this conserved exchange unlikely bears functional consequences. Alignments of the Apiaceae polypeptides with FHTs from other plant families recognized some of the exchanges conserved in FNS I also in these FHTs, which, however, cannot be disregarded for further studies, because these residues might negligibly affect FHT activity but essentially support FNS I activity. The most striking difference among the Apiaceae enzymes is a C-terminal triplet of FHT (Gln348/Glu349 or Asp349/Trp350 or Ala350 or Val350) deleted in FNS I (Fig. 2). However, this triplet was not conserved in non(Apiaceae FHTs, presumably due to weak conservation of the entire C-terminus, and insertion of the triplet in Daucus carota FNS I did not change the activity (data not shown). Provided that FNS I has evolved from FHT the triplet which does not affect FHT or FNS I activity was likely deleted shortly after gene duplication. The remaining conserved differences between FNS I and FHT noted on the alignments are scattered over the entire sequence; chimeric proteins were therefore constructed by swapping about 40% of the C-terminal portion between parsley FHT and FNS I.

Significance of the C-terminus

Previous studies concerning the relevance of the C-terminal portion of 2-ODDs on enzyme activity did not provide a coherent picture. Deletion of six amino acids from the C-terminus of Aspergillus nidulans IPNS (Sami et al., 1997) or Streptomyces clavuligerus DAOCS (Valegard et al., 1998) significantly diminished the activities, and it was suggested that the enzymes which accommodate the active site in a β-sheet barrel use the C-terminus as a protective lid to maintain a hydrophobic environment and to enable proper co-factor binding (Lloyd et al., 1999). In case of chimeric gibberellin 20-oxidases a pronounced influence of the C-terminus on product selectivity was noticed (Lange et al., 1997). However, penicillin ring
expansion by Acremonium chrysogenum DAOCS/DACS was not affected by the C-terminal deletion of 20 amino acids (Chin et al., 2003). The specificity of Petunia hybrida FHT was also retained on C-terminal truncation of 5, 11 or 24 amino acid residues, albeit the specific activity dropped by 56 to 72.6% (Wellmann et al., 2004). The Petunia FHT activity was nearly lost, however, on deletion of 29 C-terminal amino acids (0.4% activity of wild-type) or on swapping the C-terminal portion of 52 amino acids by the corresponding sequence from Citrus unshiu FLS (0.3% activity of wild-type) without a change in specificity (Wellmann et al., 2004). Thus, the contribution of the C-terminus to enzyme activity is variable for different 2-ODDs, and this aspect was examined for parsley FHT and FNS I.

Two FHT/FNS I chimera (Pet_criChim I and II) were constructed from pYES2.1 clones harbouring fully functional Petroselinum crispum FNS I or FHT sequences (Martens et al., 2001; Martens et al., 2003) (Suppl. Fig.: 1). Pet_criChim I was composed of the N-terminal 219 amino acids of FNS I ligated to the C-terminal 149 residues of FHT, whereas in Pet_criChim II the C-terminal 146 amino acid residues of FNS I were joined to the N-terminal portion of 219 amino acids from FHT. Notably, however, amino acids 217 to 296 are highly conserved in FNS I and FHT except for position 231 in FHTs outside the Apiaceae; therefore, the last 72 amino acids of the chimera only differed from the wild-type enzymes. The constructs were overexpressed in yeast, and the FHT or FNS I activity of crude extracts was determined in standard assays employing 14C-labelled naringenin as substrate followed by TLC separation and autoradiography (Martens et al., 2003). The effects of these swapping experiments on FHT vs. FNS I activity (Fig. 3) differed considerably, because recombinant Pet_criChim I mostly retained FNS I activity converting naringenin to apigenin with little FHT side activity forming dihydrokaempferol, whereas Pet_criChim II showed weak FHT activity compared to the wild-type enzyme without a trace of FNS I activity. The data rule out an essential contribution of the C-terminal enzyme portion on FNS I activity, which is supported also by fully functional truncated FNSs I reported recently from Daucus carota, Apium graveolens and Aethusa cynapium (Gebhardt et al., 2005), although an effect of the C-terminus on activity can not be neglected without kinetic evidence. It is obvious, furthermore, that the C-terminal portion of the enzyme is not strictly required for FHT activity, but the side activity of Pet_criChim I and the suppression of Pet_criChim II suggest a significant beneficial contribution on FHT activity. Taken together, a potential gain of FNS I functionality likely requires amino acids substitution(s) in the N-terminal portion (position 1-216) of the FHT sequence.
Homology modeling and choice of mutations

The structures of some crystallized 2-ODDs have been solved and reviewed by (Clifton et al., 2006). These include mostly microbial enzymes, e.g. isopenicillin N synthase (IPNS) (Roach et al., 1995), deacetoxycephalosporin C synthase (DAOCS) (Valegard et al., 1998), clavaminic acid synthase (CAS) (Zhang et al., 2000), carbapenem synthase (CarC) (Clifton et al., 2003) or proline 3-hydroxylase (P3H) (Clifton et al., 2001) and taurine/α-ketoglutarate dioxygenase (TauD) (Elkins et al., 2002). Factor-inhibiting hypoxia-inducible factor (HIF) (Elkins et al., 2003), phytanoyl-CoA 2-hydroxylase (McDonough et al., 2005), and anthocyanidin synthase (ANS) (Wilmouth et al., 2002) are examples from mammalian and plant sources. Each of these enzymes keeps its active site iron centre in a hydrophobic environment enclosed by a double-stranded β-helix or jelly roll topology. However, the extent and the periphery of the jelly rolls may vary, and the enzymes show only little sequence similarity. Irrespective of these limitations, α-helices and β-sheets appear to be analogously assembled leading to almost identical CD spectroscopic profiles for *Petunia hybrida* FHT (Lukacin et al., 2000), *Citrus unshiu* FLS (Wellmann et al., 2002) or IPNS (Borovok et al., 1996; Durairaj et al., 1996). Alignment of the 2-ODDs that have been examined by X-ray scattering with parsley FHT and FNS I polypeptides revealed the highest sequence similarity of approximately 30% with ANS.

In an attempt to denote more closely those amino acids responsible for substrate binding model calculations were done based on the ANS structure, although a sequence similarity exceeding 30% had been postulated for reliable homology modelling (Sanchez and Sali, 1997). Nevertheless, in case of UDP-glucosyltransferases from *Sorghum bicolour*, for example, 15% similarity was sufficient (Thorsøe et al., 2005). ANS had been co-crystallized with quercetin or naringenin (Wilmouth et al., 2002; Welford et al., 2005), since the natural leucoanthocyanidin substrates are unstable. The structure of the ANS-naringenin complex (2brt) was preferred for modelling, because FHT and FNS I use naringenin as substrate. Due to little sequence similarity of FNS I and FHT with ANS in the N- and C-terminal region the model calculations are based on residue 30 to 305, excluding four short N-terminal α-helices (α-helix 1-4) and two C-terminal α-helices (α-helix 16 and 17) of ANS. ANS is characterized by a jelly roll topology (β5-β12) with long α-helical backbone (α-helix 12) as observed earlier for IPNS or DAOCS. Furthermore, the jelly roll motif of ANS is extended by two β-sheets (β-3 and β-4). Homology model regions corresponding to ANS β-3 to β-6 are represented as
almost straight loops (Fig. 4), because PDB and SWISSMODELL software use slightly different protocols for the assignment of secondary structure, but those regions adopt a similar orientation as the corresponding β-sheet of ANS. The positions of residues for iron binding are strictly conserved in the homology models generated and revealed His^{218}, His^{276} und Asp^{220} in parsley FHT or FNS I, as compared to His^{232}, His^{288} und Asp^{234} in ANS, for almost octahedral coordination in conjunction with the C1- and C2-carboxyls of 2-oxoglutarate.

Substrate binding in ANS is facilitated through π-stacking of the naringenin A-ring with Phe^{304}, corresponding to Phe^{292} in FHT or FNS I (Fig. 4). Furthermore, the 7-hydroxyl of naringenin can form a hydrogen bond with the side chain of Glu^{306}, whereas the equivalent position in FHT or FNS I is held by Asn^{294} which does not engage in hydrogen bonding. ANS fixes the B-ring of the substrate through hydrophobic interaction with Phe^{144} and hydrogen bonding of the 4′-hydroxyl to Tyr^{142}. These residues are lacking from FHT or FNS I which encode Ala^{133} and Ile^{131} (FHT) or Thr^{133} and Phe^{131} (FNS I) instead. Moreover, Lys^{213} was proposed to participate in protonation and deprotonation in ANS catalysis (Wilmouth et al., 2002), while this residue is lacking in FHT and FNS I due to a gap of three amino acids between residues 200 and 201 (Fig. 5). These data suggest a substrate binding in the active site cavity of FHT and FNS I different from that in ANS and corroborate the previous proposal of ‘α-face’-specificity of ANS and FLS versus ‘β-face’-specificity of FHT and FNS I (Martens et al., 2003; Turnbull et al., 2004; Welford et al., 2005). High affinity binding of substrate is essential in both FHT and FNS I as well as in ANS catalysis because of the radical mechanisms and is supported by the narrow substrate specificity of FHT and FNS I and absence of side reactions. Conceivably, additional side chains enforce the substrate affinity, but the low sequence similarity of FHT and FNS I with ANS and putative inaccuracy of modelled side chain orientations generally associated with homology modelling ruled out more informative docking calculations. Thus, the projection of naringenin in FHT and FNS I is shown as determined for ANS (Fig. 4).

Both FHT and FNS I were proposed to initiate the loss of the β-configurated hydrogen from carbon-3 of naringenin, and the parameters of substrate binding unlikely explain the difference in product formation. However, subtle sequence differences must determine the fate of the remaining radical. Most of the differences conserved in parsley FHT or FNS I polypeptide were recognized in the periphery except for seven residues at or close to the active site cavity, assigning the substitutions M106T, I115T, V116I, I131F, D195E, V200I,
L215V and K216R as a potential cause of FHT-to-FNS I conversion (Fig. 5). The M106T exchange concerns a flexible loop corresponding to ANS α-helix 9 near the enzyme surface, and the substitutions I115T, V116I and I131F (corresponding to Tyr142 in ANS) are part of β-sheets 3 and 4, while D195E in α-14 and V200I in β-5 are exposed to the catalytic pocket. L215V and K216R are located in β-6 close to the Fe$^{2+}$/2-oxoglutarate centre. Conservative exchanges, i.e., V200I, are commonly irrelevant for enzyme function, but the exchanges V116I and L215V were examined further because of their proximity to the sites of I115T and K216R substitutions. Homology models based on the structure of the ANS-quercetin complex suggested some additional impact of the F320Y and R326K exchanges on enzyme activity (data not shown), but the low reliability of C-terminal modelling and the results obtained with Pet_criChim I and II excluded these residues from further investigations.

**Site-directed mutagenesis**

Each of the amino acids Met$^{106}$, Ile$^{115}$, Val$^{116}$, Ile$^{131}$, Asp$^{195}$, Leu$^{215}$ and Lys$^{216}$ conserved in FHTs was independently replaced by the corresponding amino acid found in the parsley FNS I sequence. Single and double mutants (M2-8; Tab. 1) were constructed from Pet_criFHT-pYES2.1 and used as templates to generate multiple mutants (M9-15, Tab. 1). For all single and double mutants (M2-8; Tab. 1), the expression in yeast cells resulted in extracts with FHT activity and without any significant FNS I activity. Consequently, the substitution of one or two amino acids is insufficient to shift the naringenin 3β-hydroxylase activity towards flavone (apigenin) formation. Two of the triple mutants harbouring M106T-I131F-D195E or I131F-L215V-K216R substitutions (M9 and M10) showed reduced FHT activity in comparison to the wild-type parsley FHT concomitant with the formation of a second product that was distinguished by TLC (Fig. 6a). This product was identified as apigenin by co-chromatography with a reference sample in three solvent systems (Martens et al., 2001). However, the triple mutant D195E-L215V-K216R (M11; Fig. 6a) did not gain FNS I activity, which emphasizes the essential role of Phe$^{131}$ for FNS I activity, although this substitution on its own (M3; Tab.1) was inefficient. Four or five substitutions including I131F were introduced in mutants 12-14 (Fig. 6a), which predominantly exhibited FHT activity with FNS I side-activity. Finally, the full set of seven mutations inferred from homology modelling was introduced in FHT (M15; Fig. 6a). This recombinant mutant enzyme showed primarily FNS I activity, albeit at a reduced level as compared to wild-type FNS I, with very little residual FHT activity detected after two-dimensional TLC in two solvent systems (Fig. 6b).
It is thus obvious that amino acid residues in the N-terminus or beyond residue 305 also contribute to FNS I activity. Swapping of the C-terminal domain of parsley FNS I by that of FHT had introduced some FHT activity suggesting FHT-relevant residues in that region. Alignments of published FNS I and FHT sequences identified Asp\textsuperscript{331} as strictly conserved in FHTs from Apiaceae and other plants, which is replaced by His in FNS I. The D331H substitution in *Pet_criFHT* (M16; Tab. 1) and *Pet_criChimI* (M17) confirmed the importance of this residue for FHT activity, because recombinant M15 displayed no enzymatic activity (data not shown) and M17 retained FNS I activity but lost the residual FHT activity (Fig. 6b).

**Discussion**

Flavonoids are abundant plant secondary metabolites which have been reported even from primitive taxa, such as the liverworts (Feld et al., 2003) and the horse tails (Oh et al., 2004). Their classification is based on the flavane skeleton and comprises a spectrum of compounds with flavones, flavonols and anthocyanins as major components. The principles of flavonoid biosynthesis have been thoroughly studied regarding the biochemistry, genetics and molecular biology, but there is little information concerning the evolution of committed enzymes (Harborne and Williams, 2000). Sessile plants have to cope with multiple environmental changes which act as a driving force for the adaptation and evolution of enzymes. This process is believed to follow basically one of two routes. An existing structural gene may change and gain the capability of encoding an enzyme with broader substrate/product specificity or multifunctionality. Alternatively, gene duplication can lead to cumulative mutations in one of the copies due to relaxed functional constraints, and often those copies are eliminated later after pseudogenization. In some instances, however, mutated copies might be retained provided that the expression is of particular benefit, such as dosage effects, subfunctionalization or the creation of a completely new function (Hughes, 1994; Lynch and Force, 2000; Ober, 2005).

Both concepts received support from investigations on the enzymology of secondary metabolites. For example, multifunctional 2-ODDs or terpene synthases are able to catalyze more than one step of a given biosynthetic pathway (Steele et al., 1998; Prescott, 2000). FLS from *Citrus unshiu* or ANS from *Arabidopsis thaliana* and *Gerbera hybrida* exhibit several activities *in vitro*, although the significance *in vivo* remained uncertain (Lukacin et al., 2003; Martens et al., 2003; Turnbull et al., 2004). On the other hand, duplications have been documented for 2-ODDs of glucosinolate biosynthesis in *Arabidopsis thaliana* and various
enzymes of flavonoid biosynthesis, such as CHS from *Gerbera hybrida* and *Ipomoea*, CHI and DFR from *Lotus* and DFR from *Ipomoea* (Helariutta et al., 1996; Hoshino et al., 2001; Kliebenstein et al., 2001; Shimada et al., 2003; Shimada et al., 2005). The phenomenon of gene duplication is not restricted to secondary metabolism, since genes of primary metabolism have also been recruited, *i.e.* deoxyhypusin synthase for the evolution of homospermidine synthase catalyzing the first committed step in pyrrolizidine alkaloid biosynthesis (Ober and Hartmann, 1999). Also the evolution of FNS I from FHT by gene duplication was suggested, but the prime ancestor gene remains to be identified (Martens et al., 2003; Gebhardt et al., 2005). Due to sequence similarity and substrate specificity flavonoid dioxygenases were grouped into 2-ODDs with low substrate specificity which attack the “α-face” of the substrate such as ANS and FLS, and 2-ODDs with high substrate specificity like FHT and FNS I which attack the “β-face” (Lukacin et al., 2003; Martens et al., 2003; Turnbull et al., 2004). Both FHT and FNS I withdraw the β-configurated hydrogen from carbon-3 of naringenin, but then proceed on different routes despite their high sequence similarity (Fig. 7). FHT catalyses the 3β-hydroxylation through a rebound process, whereas FNS I affords the syn-elimination of hydrogen from carbon-2 in a cage-like setting without intermediate hydroxylation (Fig. 7b). The proposed FNS I reaction clearly differs from the mechanisms assumed for FLS or ANS, which likely hydroxylate carbon-3 or -2 of the substrate followed by antiperiplanar water elimination as indicated by small amounts of dihydrokaempferol and kaempferol byproducts (Welford et al., 2001), but is compatible with the previous finding that FNS I neither converts 2-hydroxynaringenin nor dihydroflavonols to flavones (Britsch, 1990; Martens et al., 2003). Indirect experimental support for the syn-elimination mechanism was provided recently by incubations of ANS with naringenin diastereomers (Welford et al., 2005), because the selectivity of ANS for substrates with a particular C-2 stereochemistry is greatly diminished in the absence of a C-3 hydroxygroup. Mostly dihydrokaempferol and kaempferol, besides traces of apigenin, were formed from (2S)-naringenin, whereas almost equivalent amounts of dihydrokaempferol and apigenin with little kaempferol resulted from unnatural (2R)-naringenin which exposes the 2α- and 3α-configurated hydrogens to the catalytic ferryl species in ANS (Welford et al., 2005) (Fig. 8a).

The crystal complex revealed that the 3α-hydrogen is closer to the ferryl species and presumably attacked first to release apigenin by syn-elimination (Welford et al., 2005). Overall, the precision of naringenin fixation with respect to the ferryl species in the active site pocket of ANS determines whether syn-elimination is preferred over hydroxylation. These findings can be extrapolated to the FNS I and FHT reactions. Both enzymes exclusively
accept flavanone substrates exposing a β-face hydrogen (Lukacin et al., 2003; Martens et al., 2003; Turnbull et al., 2004). Following the argument for apigenin formation from (2R)-naringenin by ANS-catalysed syn-elimination (Welford et al., 2005), the stereoconfiguration at C-2 of (2R)-naringenin interferes with FNS I catalysis and, in fact, FNS I does not convert (2R)-naringenin (Britsch, 1990). Thus, FNS I and FHT conceivably approach the common substrate (2S)-naringenin from the opposite site of the ring plane (Fig. 8b) as compared to ANS (Fig. 8a), which requires a mirror-image orientation of substrate and active site residues. Two combinations of I131F with M106T/ D195E (M8) or L215V/ K216R (M9) have been shown to confer FNS I–side activity, hence; these substitutions likely influence product specificity by adjusting the substrate position rather than actively participating in the reaction mechanism, e. g. through acidic or basic amino acids. Necessarily carbon-3 should be closer to the ferryl species than carbon-2 for hydroxylation by FHT, whereas the protons at carbons-2 and –3 may be equaly distant from the ferryl species in FNS I. Overall, the conserved differences in FNS I appear to fit the substrate into the active site pocket with maximal proximity of H-2 and βH-3 to the catalytic ferryl species. The essential voluminous Phe^{131} as compared to Ile^{131} in FHT supports the assumption.

FHT and FNS I are phylogenetically closely related and adopt a tertiary structure similar to ANS (Martens et al., 2001; Martens et al., 2003; Gebhardt et al., 2005). Although the detailed effects of selective amino acid substitutions on the overall parsley FHT structure are unknown, this report assigns for the first time those residues proximal to the active site which control FHT and FNS I activities. Obviously minor mutations of parsley FHT are sufficient to shift the activity, and significant FNS I activity was conferred already by a triple mutation (M9 and M10), which still retained the capacity for dihydrokaempferol formation (FHT activity). Concomitantly, however, a severe loss in specific enzyme activity was observed. The replacement of seven amino acids caused a nearly complete change towards FNS I activity. The process accomplished here by site-directed mutagenesis defines the minimal conditions for directed evolution in vivo to broaden the flavonoid spectrum. Plants following this route like the Apiaceae might have gained the capacity of flavone accumulation without loosing their flavonols provided that gene duplication had occurred. It is likely that the efficiency and selectivity of the newly formed FNS I has improved with time through additional mutations and concomitant with the complete loss of FHT activity in the gene copy. The ease of change of function by only three mutations of FHT suggests that flavone biosynthesis may have evolved independently on this route more than once, however, other
enzymes exhibiting FNS I activity have not been observed outside the Apiaceae. The accumulation of flavones conceivably provides an advantage to the plant, because expression of FNS I has been maintained in the Apiaceae, and other plants developed FNS II for the same purpose.

The capacity to form flavonoids is supposed to have developed gradually, because the first flavonoid enzymes were probably not as effective or selective as today, and the initially low flavonoid concentrations likely served in plant signalling rather than UV protection or defence (Stafford, 1991). In any case, the conservation of flavone biosynthesis indicates an advantage which eventually led to flavone concentrations sufficient also for UV protection of the plant, and the environmental impact likely furthered the evolution of FNS I in Apiaceae (Logemann et al., 2000; Solovchenko and Schmitz-Eiberger, 2003). The early ontogenetic expression of FNS I in parsley and the gradual replacement of flavonols through flavones in more advanced members of the Apiaceae (Harborne, 1971; Gebhardt et al., 2005) support this assumption. Advantage of functional FNSs for plant families, and the identification of FNS II in non-Apiceae is a clear indication of convergent evolution. It remains to be established whether FNS I has also evolved in non-Apiceae, taking into account that few mutations are sufficient to confer this activity on a FHT. The search for non-Apiceae FNS I is an interesting challenge.

While flavonols and flavones have been isolated from spermatophytic and primitive plants, anthocyanidins are confined to the more advanced gymnosperms and angiosperms. This seems to suggest that FHT and FNS I developed early followed much later by FLS and ANS (Prescott and John, 1996). However, Prescott and John (1996) also excluded FHT as a direct progenitor of ANS, because of low sequence similarity and divergent gene structures. There is no experimental evidence so far for an early evolution of FNS I, because the confinement to Apiaceae and the high sequence similarity with FHT suggest a fairly recent duplication event. It appears more likely that flavonoid 2-ODDs developed from a common multifunctional ancestor gene, because FLS and ANS show low substrate specificity which could be attributed to either incomplete or spreading evolution. The low stringency might be explained by channelling of substrates in multienzyme complexes (Winkel-Shirley, 2001) releasing the evolutionary pressure for enzymes of narrow substrate specificity. Under these premises, the physiological function of ANS must be re-evaluated, because in vitro ANS predominantly converted leucoanthocyanidin to dihydroquercetin, (2S)-naringenin to dihydrokaempferol and
dihydroquercetin to quercetin (Welford et al., 2001). The lack of anthocyanidins in less advanced plants could thus be a consequence of poor complex formation rather than lack of ANS. The capability of catalyzing several steps in the flavonoid pathway might furthermore qualify ANS also as a progenitor candidate of other flavonoid 2-ODDs, and it is essential to determine the evolutionary distance of the various flavonoid 2-ODDs. The functional flexibility of these 2-ODDs by very few mutations was demonstrated in this study and highlights the evolutionary importance of 2-ODDs for the introduction of new enzymatic functions.

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Experimental procedures

Yeast strains and growth conditions
Saccharomyces cerevisiae INV Sc1 (Invitrogen, Karlsruhe, Germany) was used for standard cloning and over expression. Media and growth conditions are described elsewhere ((Martens et al., 2003). All plasmids for chimeric FHT/FNS I and mutagenesis were constructed from pYES2.1 that contains the Petroselinum crispum FHT (Pet_criFHT) or FNS I (Pet_criFNSI; (Martens et al., 2001; Martens et al., 2003)).

Molecular techniques and cloning of 2-ODDs
Plasmid DNA was isolated as described in (Engebrecht et al., 2001). For sequencing DNA was further purified by being passed through a Nucleo Spin column according to manufacturer’s instructions (Machery & Nagel, Düren, Germany). Restriction digestions were performed as described by the enzyme suppliers (MBI Fermentas, Leon Roth, Germany). Yeast transformations were performed according to EasyComp protocol (Invitrogen, Karlsruhe, Germany). Ligation reactions and agarose gel electrophorese were performed by standard procedures (Sambrook and Russel, 2001). Further putative FHT and FNS I cDNA clones were isolated and functionally verified as described in (Gebhardt et al., 2005).

Chimeric gene construction
Highly conserved regions of the FHT and FNS I gene were identified by a multiple sequence alignment of a number of 2-ODDs (Gebhardt et al., 2005). Chimeric constructs (Pet_criChim I and II) are based on functionally verified Pet_criFNSI and FHT pYES2.1 clones. 

BamHI is conserved between T219 and D220 in both sequences and was used in combination with XbaI (located 3’ of the insert in the multiple cloning site of pYES2.1) to digest the cDNA clones, resulting in a long (pYES2.1 and N-terminal part of the insert) and a short (C-terminal part of the insert) fragment. All fragments were gel purified after restriction digestion, isolated from the gel via NucleoSpin® Extract kit and added to the ligation reaction. The long fragment of the FNS I digest was combined with the short fragment of the FHT digest resulting in Pet_criChim I and vice versa resulting in Pet_criChim II as illustrated in Suppl. Fig.: 1. Full length sequencing of the inserts confirmed successful ligation of the fragments and intact open reading frames for both chimerics.

Site directed mutagenesis of Pet_criFHT

Single and multiple amino acids substitutions were generated by site-directed mutation of Pet_criFHT or its previously mutated variants in pYES2.1 by Stratagene QuickChange® and QuickChange® Multi System according to the manufacturer’s instructions (Stratagene, La Jolla, CA, USA). All primers and templates for mutagenesis are listed in Suppl. Tab. 2.

In detail, the plasmid Pet_criFHT/pYES2.1 (Martens et al., 2003) was used as primary template together with a respective mutation primer (Suppl. Tab. 2). For additional specific mutations the confirmed mutants of previous rounds were used as indicated in Tab. 1. Some mutagenic oligonucleotides were designed with a silent mutation to generate a new or destroy a restriction site (Suppl. Tab. 2) to facilitate the identification of clones carrying the desired mutation. All mutant FHTs were sequenced in full length to ensure the correct residue was changed and to confirm that no other unintended mutation was introduced (MWG-Biotech, Ebersberg, Germany).

Expression of cloned genes and analysis of catalytic properties

Enzymatic activities of the wild type, chimeric and mutated proteins were determined as previously described by heterologous expression in yeast (Gebhardt et al., 2005) with 500 µg and 1000 µg total protein respectively as double tests. Protein concentrations were determined according to Bradford (Bradford, 1976) with bovine serum albumin as a standard.

Sequence comparison
Related sequences were initially detected by BLAST and PSI-BLAST (Altschul et al., 1997) analysis. Multiple sequence alignments were generated with CLUSTAL W algorithm (Thompson et al., 1994)

*Homology Modelling*

In order to identify putative amino acids responsible for the different catalytic behaviour of FNS I and FHT homology modelling was performed with respect to *Petroselinum crispum* FNS I and FHT. Homology models were generated using the web-based SWISSMODEL server (Schwede et al., 2003). The crystal structure of ANS in complex with Fe$^{2+}$, 2-oxoglutarate and naringenin (pdb-entry 2brt) was kindly provided in advance by the authors (Welford et al., 2005) and served as template structure. Even though the homology models obtained were truncated with respect to the N- as well as the C-terminus these models clearly represented the substrate binding pocket and, accordingly, allowed for the structural location of sequence mismatches within the binding cavities. As SWISSMODEL recognizes only protein atoms, NAR, 2-oxoglutarate and the iron ion were added to the models at similar positions as observed in the template structure. However, this step has to be regarded with some care, as the binding mode of NAR to ANS is certainly different from FNS I and FHT. Thus, the insertion of the substrate was performed to evaluate putative interaction sites between naringenin and its binding pockets. Amino acid residues of the two models involved in iron- and co-substrate binding were manually adjusted using the program O (Jones et al., 1991). Figures were created by means of Pymol (DeLano, 2002).

*References*

Altschul SF, Madden TL, Schäffer AA, Zhang J, Zhang Z, Miller W, Lipman DJ (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res. 25: 3389-3402

Borovok I, Landman O, Kreisberg-Zakarin R, Aharonowitz Y, Cohen G (1996) Ferrous active site of isopenicillin N synthase: genetic and sequence analysis of the endogenous ligands. Biochemistry 35: 1981-1987

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of proteindye binding. Analytical Biochemistry 72: 248-254
Britsch L (1990) Purification and characterisation of flavone synthase I, a 2-oxoglutarate-dependent desaturase. Arch. Biochem. Biophys. **282**: 152-160

Chin HS, Sim J, Seah KI, Sim TS (2003) Deacetoxycephalosporin C synthase isozymes exhibit diverse catalytic activity and substrate specificity. FEMS Microbiol. Lett. **218**: 251-257

Clifton IJ, Doan LX, Sleemann MC, Topf M, Suzuki H, Wilmouth RC, Schofield CJ (2003) Crystal Structure of Carbapenem Synthase (CarC). J. Biol. Chem. **278**: 20843-20850

Clifton IJ, Hsueh L-C, Baldwin JE, Harlos K, Schofield CJ (2001) Structure of proline 3-hydroxylase. Evolution of the family of 2-oxoglutarate dependent oxygenases. European Journal of Biochemistry **268**: 6625-6636

Clifton IJ, McDonough MA, Ehrismann D, Kershaw NJ, Granatino N, Schofield CJ (2006) Structural studies on 2-oxoglutarate oxygenases and related double-stranded β-helix fold proteins. Journal of Inorganic Biochemistry **100**: 644-669

DeLano WL (2002) The PyMOL Molecular Graphics System. In [http://www.pymol.org](http://www.pymol.org).

DeLano Scientific, Palo Alto, CA, USA

Durairaj M, Leskiw BK, Jensen SE (1996) Genetic and biochemical analysis of the cysteinyl residues of isopenicillin N synthase from *Streptomyces clavuligerus*. Can. J. Microbiol. **42**: 870-875

Elkins JM, Hewitson KS, McNeill LA, Seibel JF, Schlemminger I, Pugh CW, Ratcliffe PJ, Schofield CJ (2003) Structure of Factor-inhibiting Hypoxia-inducible Factor (HIF) Reveals Mechanism of Oxidative Modification of HIF-1α. J. Biol. Chem. **278**: 1802-1806

Elkins JM, Ryle MJ, Clifton IJ, Hotopp JCD, Lloyd JS, Burzlaff NI, Baldwin JE, Hausinger RP, Roach PL (2002) X-ray crystal structure of *Escherichia coli* taurine/α-ketoglutarate dioxygenase complexed to ferrous iron and substrates. Biochemistry **41**: 5185-5192

Engebrect J, Brent R, Kaderbhai MA (2001) Minipreps of Plasmid DNA. In EW Harkins, ed, Current Protocols in Molecular Biology. John Wiley & Sons, Inc.

Feld H, Zapp J, Becker H (2003) Secondary metabolites from the liverwort *Tylimanthus renifolius*. Phytochemistry **64**: 1335-1340

Gebhardt Y, Witte S, Forkmann G, Lukacin R, Matern U, Martens S (2005) Molecular evolution of flavonoid dioxygenases in the family Apiaceae. Phytochemistry **66**: 1273-1284
Harborne JB (1971) Flavonoid and phenylpropanoid patterns in the Umbelliferae. In The Biology and Chemistry of the Umbelliferae. Academic Press, London, UK, pp 293-314

Harborne JB, Baxter H (1999) The Handbook of Natural Flavonoids. In The Handbook of Natural Flavonoids, Vol 2. Wiley-VCH Verlag GmbH, Weinheim

Harborne JB, Williams C (2000) Advances in flavonoid research since 1992. Phytochemistry 55: 481-504

Harborne JB, Williams CA (1972) Flavonoid patterns in the fruits of the Umbelliferae. Phytochemistry 11: 1741-1750

Helariutta Y, Kotilainen M, Elomaa P, Kalkkinen N, Bremer K, Teeri TH, Albert VA (1996) Duplication and functional divergence in the chalcone synthase gene family of Asteraceae: evolution with substrate change and catalytic simplification. Proc. Natl. Acad. Sci. U. S. A. 93: 9033-9038

Hogan DA, Smith SR, Saari EA, McCracken J, Hausinger RP (2000) Site-directed Mutagenesis of 2,4-Dichlorophenoxyacetic Acid/a-Ketoglutarate Dioxygenase. The Journal of Biological Chemistry 275: 12400-12409

Hoshino A, Johzuka-Hisatomi Y, Iida S (2001) Gene duplication and mobile genetic elements in the morning glories. Gene 265: 1-10

Hughes AL (1994) The evolution of functionally novel proteins after gene duplication. Proc. R. Soc. London B. Biol. Sci. 256: 119-124

Jones TA, Zou JY, Cowan SW, Kjeldgaard M (1991) Improved methods for building protein models in electron density maps and the location of errors in these models. Acta Cryst. A 47: 110-119

Kliebenstein DJ, Lambrrix VM, Reichelt M, Gershenzon J, Mitchell-Olds T (2001) Gene Duplication in the Diversification of Secondary Metabolism: Tandem 2-Oxoglutarate–Dependent Dioxygenases Control Glucosinolate Biosynthesis in Arabidopsis. The Plant Cell 13: 681–693

Lange T, Kegler C, Hedden P, Phillips AL, Graebe JE (1997) Molecular characterisation of gibberellin 20-oxidases. Structure-function studies on recombinant enzymes and chimaeric proteins. Physiol. Plant 100: 543-549

Lloyd MD, Lee H-J, Harlos K, Zhang Z-H, Baldwin JE, Schofield CJ, Charnock JM, Garner CD, Hara T, Scheltinga ACTv, Valegard K, Viklund JAC, Hajdu J, Andersson I, Danielsson A, Bhikhabhai R (1999) Studies on the Active Site of Deacetoxycephalosporin C Synthase. J. Mol. Biol. 287: 943-960
Logemann E, Tavernaro A, Schulz W, Somssich IE, Hahlbrock K (2000) UV light selectively coinduces supply pathways from primary metabolism and flavonoid secondary product formation in parsley. Proc. Natl. Acad. Sci. U. S. A. 97: 1903-1907

Lukacin R, Britsch L (1997) Identification of strictly conserved histidine and arginine residues as part of the active site in Petunia hybrida flavanone 3beta- hydroxylase. European Journal of Biochemistry 249: 748-757

Lukacin R, Urbanke C, Gröning I, Matern U (2000) The monomeric polypeptide comprises the functional flavanone 3ß-hydroxylase from Petunia hybrida. FEBS letters 467: 353-358

Lukacin R, Wellmann F, Britsch L, Martens S, Matern U (2003) Flavonol synthase from Citrus unshiu is a bifunctional dioxygenase. Phytochemistry 62: 287-292

Lynch M, Force A (2000) The probability of duplicate gene preservation by subfunctionalization. Genetics 154: 459-473

Martens S, Forkmann G, Britsch L, Wellmann F, Matern U, Lukacin R (2003) Divergent evolution of flavonoid 2-oxoglutarate-dependent dioxygenases in parsley. FEBS letters 544: 93-98

Martens S, Forkmann G, Matern U, Lukacin R (2001) Cloning of parsley flavone synthase I. Phytochemistry 58: 43-46

Martens S, Mithöfer A (2005) Flavones and flavone synthases. Phytochemistry 66: 2399-2407

McDonough MA, Kavanagh KL, Sears DB, Oppermann U, Schofield CJ (2005) Structure of human phytanoyl-CoA 2-hydroxylase identifies molecular mechanisms of Refsum disease. J. Biol. Chem. 280: 41101-41110

Myllyharju J, Kivirikko KI (1997) Characterization of the iron- and 2-oxoglutaratebinding sites of human prolyl 4-hydroxylase. EMBO J. 16: 1173-1180

Ober D (2005) Seeing double: gene duplication and diversification in plant secondary metabolism. Trends in Plant Science 10: 444-449

Ober D, Hartmann T (1999) Homospermidine synthase, the first pathway-specific enzyme of pyrrolizidine alkaloid biosynthesis, evolved from deoxyhypusine synthase. Proc. Natl. Acad. Sci. U. S. A. 96: 14777-14782

Oh H, Kim DH, Cho JH, Kim YC (2004) Hepatoprotective and free radical scavenging activities of phenolic petrosins, flavonoids isolated from Equisetum arvense. J. Ethnopharmacol. 95: 421-424
Prescott AG (2000) Two-oxoacid-dependent dioxygenases: inefficient enzymes or evolutionary driving force. In JTRe al., ed, Evolution of Metabolic Pathways. Elsevier Science Ltd., pp 249-284

Prescott AG, John P (1996) Dioxygenases: Molecular structure and role in plant metabolism. Annu. Rev. Plant Physiol. Plant Mol. Biol. 47: 245-271

Prescott AG, Lloyd MD (2000) The iron(II) and 2-oxoacid-dependent dioxygenases and their role in metabolism. Nat. Prod. Rep. 17: 367-383

Roach PL, Clifton IJ, Fulop V, Harlos K, Barton GJ, Hajdu J, Andersson I, Schofield CJ, Baldwin JE (1995) Crystal structure of isopenicillin N synthase is the first from a new structural family of enzymes. Nature 375: 700-7004

Roach PL, Clifton IJ, Shibata N, Hajdu J, Schofield CJ, Baldwin JE (1997) Structure of isopenicillin N synthase complexed with substrate and the mechanism of penicillin formation. Nature 387: 827-830

Sambrook J, Russell DW (2001) Molecular cloning - A laboratory manual, Ed 3. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York

Sami M, Brown TJN, Roach PL, Schofield CJ, Baldwin JE (1997) Glutamine-330 is not essential for activity in Isopenicillin N-synthase from Aspergillus nidulans. FEBS Letters 405: 191-194

Sanchez R, Sali A (1997) Advances in comparative protein-structure modelling. Curr. Opin. Struct. Biol. 7: 206–214

Schwede T, Kopp J, Guex N, Peitsch MC (2003) SWISS-MODEL: an automated protein homology-modeling server. Nucleic Acids Research 31: 3381-3385

Shimada N, Aoki T, Sato S, Nakamura Y, Tabata S, Ayabe S (2003) A cluster of genes encodes the two types of chalcone isomerase involved in the biosynthesis of general flavonoids and legume-specific 5-deoxy(iso)flavonoids in Lotus japonicus. Plant Physiol. 131: 941-951

Shimada N, Sasaki R, Sato S, Kaneko T, Tabata S, Aoki T, Ayabe S-i (2005) A comprehensive analysis of six dihydroflavonol 4-reductases encoded by a gene cluster of the Lotus japonicus genome. Journal of Experimental Botany 56: 2573–2585

Solovchenko A, Schmitz-Eiberger M (2003) Significance of skin flavonoids for UV-B-protection in apple fruits. J. Exp. Bot. 54: 1977-1984

Stafford HA (1991) Flavonoid Evolution: An Enzymic Approach. Plant Physiol. 96: 680-685

Steele CL, Crock J, Bohlmann J, Croteau R (1998) Sesquiterpene synthases from grand fir (Abies grandis). Comparison of constitutive and wound-induced activities, and cDNA...
isolation, characterization, and bacterial expression of delta-selinene synthase and gamma-humulene synthase. J. Biol. Chem. 273: 2078-2089

Thompson JD, Higgins DG, Gibson TJ (1994) CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res. 22: 4673-4680

Thorsøe KS, Bak S, Olsen CE, Imberty A, Breton C, Møller BL (2005) Determination of Catalytic Key Amino Acids and UDP Sugar Donor Specificity of the Cyanohydrin Glycosyltransferase UGT85B1 from Sorghum bicolor. Molecular Modeling Substantiated by Site-Specific Mutagenesis and Biochemical Analyses. Plant Physiology 139: 664–673

Turnbull JJ, Nakajima J-i, Welford RWD, Yamazaki M, Saito K, Schofield CJ (2004) Mechanistic Studies on Three 2-Oxoglutarate-dependent Oxygenases of Flavonoid Biosynthesis. The Journal of Biological Biochemistry 279: 1206–1216

Valegard K, Scheltinga ACv, Lloyd MD, Hara T, Ramaswamy S, Perrakis A, Thompson A, Lee HJ, Baldwin JE, Schofield CJ, Hajdu J, Andersson I (1998) Structure of a cephalosporin synthase. Nature 394: 805-809

Welford RWD, Clifton IJ, Turnbull JJ, Wilson SC, Schofield CJ (2005) Structural and mechanistic studies on anthocyanidin synthase catalysed oxidation of flavanone substrates: the effect of C-2 stereochemistry on product selectivity and mechanism. Org. Biomol. Chem. 3: 3117-3126

Welford RWD, Turnbull JJ, Claridge TDW, Prescott AG, Schofield CJ (2001) Evidence for oxidation at C-3 of the flavonoid C-ring during anthocyanin biosynthesis. Chem. Commun.: 1828-1829

Wellmann F, Lukacin R, Moriguchi T, Britsch L, Schlitz E, Matern U (2002) Functional expression and mutational analysis of flavonol synthase from Citrus unshiu. European Journal of Biochemistry 269: 4134-4142

Wellmann F, Matern U, Lukacin R (2004) Significance of C-terminal sequence elements for Petunia flavanone 3ß-hydroxylase activity. FEBS letters 561: 149-154

Wilsmouth RC, Turnbull JJ, Welford RWD, Clifton IJ, Andrea G. Prescott (2002) Structure and Mechanism of Anthocyanidin Synthase from Arabidopsis thaliana. Structure 10: 93–103

Winkel-Shirley B (2001) Flavonoid Biosynthesis. A Colorful Model for Genetics, Biochemistry, Cell Biology, and Biotechnology. Plant Physiology 126: 485–493
Zhang Z, Ren J, Stammers DK, Baldwin JE, Harlos K, Schofield CJ (2000) Structural origins of the selectivity of the trifunctional oxygenase clavaminic acid synthase. Nature Structural Biology 7: 127-133
**Figure 1:** Schematic flavonoid pathway

**Figure 2:** Schematic overview of conserved substitutions in FHT and FNS I sequences. Amino acids which were assigned by homology modelling to the active site are underlined. I312V is shown in grey, because Ang_arcFNS I preserved Valin in this position. The BamHI restriction site used for construction of Pet_criChimI and II is marked by an arrow. Solid bars indicate sequence regions that are either identical or not conserved and open bar regions mark the position of amino acids responsible for cofactor binding, conserved in all 2-ODDs (Lukacin and Britsch 1997).

**Figure 3:** Conversion of $^{14}$C-labelled (2S)-naringenin with crude protein extracts from yeast transformed with Pet_criFNS I, Pet_criFHT, Pet_criChimI or Pet_CriChimII. Enzyme assays were carried out as described previously (Martens et al. 2003). Substrate and product positions vary in the presentation, because the assays were separated on different thin-layer plates. However, products were unequivocally identified by co-chromatography with authentic standards as: 1: apigenin; 2: naringenin; 3: dihydrokaempferol (Martens et al., 2003).

**Figure 4:** (a) Structure of ANS complexed with naringenin (2brt). (Welford et al. 2005). The jelly roll motif ($\beta$-5 to $\beta$-12) and its extending $\beta$-sheets are represented in yellow. (b) Homology model of Pet_criFHT based on the ANS-naringenin-structure (2brt). (c) Homology model of Pet_criFNS I based on the ANS-naringenin-structure (2brt). $\alpha$-Helices and $\beta$-sheets in the homology models were numbered according to the model template. Due to the limited sequence similarities and mechanistic differences between template and FNS I or FHT the substrate naringenin likely adopts spatially different positions in the active site pockets. The substrate was not fitted in (b) and (c), but the models clearly resolve the conserved differences between FNS I and FHT concerning the active site residues. Residues conserved differently in Pet_criFHT and Pet_criFNS I and chosen for mutagenic studies are shown as sticks.

**Figure 5:** Alignment of Ara_thaANS and partial FNS I and FHT sequences.
Similar amino acids are indicated by dots, identical amino acids by stars. Colored stars mark conserved amino acids necessary for co-factor or substrate binding. Conserved substitutions of FNS I and FHT are shown in pink (positioned in the active site) and green (peripheral
position). The BamHI restriction site used for the construction of Pet_criChimI and II is indicated by an arrow. Secondary structure elements of ANS are underlined; α-helices are labelled in black, 3₁₀-helices in bold grey, β-sheets of the jelly roll motif in bold black (Wilmouth et al. 2002).

**Figure 6:** Enzyme assays with crude protein extracts from yeast transformed with Pet_criFNS I, Pet_criFHT or Pet_criFHT mutants were carried out as described previously (Martens et al. 2003). Substrate and product positions vary in the presentation, because the assays were separated on different thin-layer plates. However, Flavonoids were unequivocally identified through co-chromatography with authentic standards. 1, apigenin; 2, naringenin; 3, dihydrokaempferol. (a) Radio scan of one dimensional TLC separation in 30 % acetic acid. (b) Radio scan of two dimensional TLC separation in CAW and 30 % acetic acid.

**Figure 7:** Reaction mechanism of (a) FHT and (b) FNSI. Depending on the substrates orientation syn-elimination and formation of flavones may proceed via a radical mechanism with initial attack at either C-3 or C-2 or both simultaneously in a concerted mechanism.

**Figure 8:** (a) Positioning of naringenin in the active site of ANS (Welford et al. 2005) and (b) proposed positioning of naringenin in the active site of FNS I.

**Supplement Figure 1:** Construction of chimeric FNS I/FHT from parsley.
Table 1: *Pet_cri*FHTs and preferential product formation.

| No. | Template     | Mutations                | Proportion of products | DHK : Ap |
|-----|--------------|--------------------------|------------------------|----------|
| 1   | *Pet_cri*FHT | wildtype                 |                        | 100 : 0  |
| 2   | *Pet_cri*FHT | M106T                    |                        | 100 : 0  |
| 3   | *Pet_cri*FHT | I115T                    |                        | 100 : 0  |
| 4   | *Pet_cri*FHT | I131F                    |                        | 100 : 0  |
| 5   | *Pet_cri*FHT | D195E                    |                        | 100 : 0  |
| 6   | *Pet_cri*FHT | I115T, V116I             |                        | 100 : 0  |
| 7   | *Pet_cri*FHT | I131F, D195E             |                        | 100 : 0  |
| 8   | *Pet_cri*FHT | L215V, K216R             |                        | 100 : 0  |
| 9   | Mutant 6     | M106T, I131F, D195E      |                        | 85 : 15  |
| 10  | Mutant 3     | I131F, L215V, K216R      |                        | 78 : 22  |
| 11  | Mutant 4     | D195E, L215V, K216R      |                        | 100 : 0  |
| 12  | Mutant 6     | I115T, V116I, I131F, D195E |                    | 76 : 24  |
| 13  | Mutant 6     | I131F, D195E, L215V, K216R |                  | 69 : 31  |
| 14  | Mutant 8     | M106T, I115T, V116I, I131F, D195E |     | 66 : 34  |
| 15  | Mutant 13    | M106T, I115T, V116I, I131F, D195E, L215V, K216R | | 18 : 82  |
| 16  | *Pet_cri*FHT | D331H                    | inactive               |          |
| 17  | *Pet_cri*ChimI | D331H                  |                        | 0 : 100  |

* *Pet_cri*FNS wildtype

Standard 2-ODD activity assays were carried out in duplicate with 5000 dpm $^{14}$C-labelled naringenin (approximately 45 pmol) and 500 or 1000 µg total protein as described previously (Martens et al. 2003). The substrate specificity of wildtype and mutant FHTs is compared by the ratio of dihydrokaempferol (FHT activity) to apigenin (FNS I activity) formation, which together represent the total product in each assay. Mutant enzymes showing both enzymatic activities were verified by expression of another clone carrying the same mutation and repeated activity assays.

* wildtype FNS produced exclusively apigenin from (2S)-naringenin.
Figure 1:

3 Malonyl-CoA  4-Coumaroyl-CoA

\[ \text{R} = \text{H: Naringenin} \]
\[ \text{R} = \text{OH: Erodicetiol} \]

\[ \text{R} = \text{H: Apigenin} \]
\[ \text{R} = \text{OH: Luteolin} \]

\[ \text{R} = \text{H: Kaempferol} \]
\[ \text{R} = \text{OH: Quercetin} \]

\[ \text{R} = \text{H: Naringenin} \]
\[ \text{R} = \text{OH: Erodicetiol} \]

\[ \text{R} = \text{H: Apigenin} \]
\[ \text{R} = \text{OH: Luteolin} \]

\[ \text{R} = \text{H: Kaempferol} \]
\[ \text{R} = \text{OH: Quercetin} \]

\[ \text{R} = \text{H: Leucopelargonidin} \]
\[ \text{R} = \text{OH: Leucocyanidin} \]

\[ \text{R} = \text{H: Pelargonidin 3-glucoside} \]
\[ \text{R} = \text{OH: Cyanidin 3-glucoside} \]
Figure 2:

- FHT: [Sequence Diagram]
- FNSI: [Sequence Diagram]

\[ = 10 \text{ amino acids (aa)} \]
Figure 3:

- **Pet_criFNS I**
  - Intensity vs. Position (pixel)
  - Peaks labeled as 1 and 2

- **Pet_criFHT**
  - Intensity vs. Position (pixel)
  - Peaks labeled as 1, 2, and 3

- **Pet_criChimI**
  - Intensity vs. Position (pixel)
  - Peaks labeled as 1, 2, and 3

- **Pet_criChimII**
  - Intensity vs. Position (pixel)
  - Peaks labeled as 2 and 3
**Figure 5:**

| FNS I 30 | A YMPNENIPV |
| FHT 32 | KFSDNIPV |
| 2brt 1 | -MVAVREVES LASSGISIP KEYIPPEKL KEINDVPLE KSHEGDQVPT |
| n1 | n2 | n3 | n4 |
| FNS I 41 | ISLAGLIDCS DGRYPSICEK IYRACKQNI FYVVRSEGRS DLSIEMTRLS |
| FHT 41 | ISLAGLIDCS WYERQSCIEK IYRACKQNI FYVVRSEGRS DLSIEMTRLS |
| 2brt 50 | ISLEKEREQD KIYSINFRCEK LALSGLOMLKLVISQIP FALEMRRVFA |
| P1 | P2 | P3 | P4 |
| FNS I 91 | REFFALPAEE KLIEDE QTVG TDGKRGGF TITLST VLQGQDD |
| FHT 91 | REFFALPAEE KLIEDE QTVG TDGKRGGF TITLST VLQGQDD |
| 2brt 100 | EEFF SLSVEE KEKYQAT DNDQAT GQIKGYG SKL ANNASGQLEW EDYFFHLAY |
| FNS I 139 | INADYSEMPK KEPGEMSTT EVYSEKLWGL GAKQELVSE AMELQKDLT |
| FHT 139 | KQADYSEMPK DPGEMSTT EVYSEKLWGL GAKQELVSE AMELQKDLT |
| 2brt 150 | REERDLIMP KTPDYSIEMAT SYYKCDLML AKTVFMAEG GLSILFQMLE |
| n5 | n6 | n7 | n8 | n9 | p1 |
| FNS I 189 | KACVDM EKV LNYEP CPQPDLELGV EAH TDVSLAFLH IMVPLG |
| FHT 189 | KACVDM DQKV LNYEP CPQPDLELGV EAH TDVSLAFLH IMVPLG |
| 2brt 200 | KEVGLEE ELMQMKINYY KP CPQPELALGV EAH TDVSLAFLH IMVPLG |
| FNS I 236 | QATRDGGKTW ITFQPVEGAV VVNLGDHGHY LSNGRFKNAD HQAVVNSSS |
| FHT 236 | QATRDGGKTW ITFQPVEGAV VVNLGDHGHY LSNGRFKNAD HQAVVNSSS |
| 2brt 250 | QLFY EG--KW VTAKCVPDSI VMHI GDTLEI LSNGKYKSIL HRGLVNKEV |
| p8 | p9 | p10 | n15 | p11 |
| FNS I 286 | KLSIATTQP AQRAYIVYQK I -------- -------- -------- |
| FHT 286 | KLSIATTQP AQRAYIVYQK I -------- -------- -------- |
| 2brt 298 | RIMAYTQFRD EREDLIEPL RENVQVPSA KFPPEPFAQH IEBLLEGKED |
| p12 | p13 | n16 | p16 / n17 |

2-Oxo DHQ

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Figure 6:

(a) Pet_criFNSI

Pet_criFHT

Pet_criM10

Pet_criM11

Pet_criM13

Pet_criM15

(b) AcOH

CAW
Figure 7:

a)  
(2S)-Naringenin  

\[ \text{Enzyme} \]

\[ \text{Fe}^{(IV)} \]

\[ \text{HO} \]

\[ \text{OH} \]

\[ \text{OH} \]

\[ \text{OH} \]

\[ \text{Fe}^{(II)} \]

\[ \text{Enzyme} \]

(2R,3R)-trans-DHK  

\[ \text{Fe}^{(II)} \]

b)  
(2S)-Naringenin  

\[ \text{Enzyme} \]

\[ \text{Fe}^{(IV)} \]

\[ \text{HO} \]

\[ \text{OH} \]

\[ \text{OH} \]

\[ \text{OH} \]

\[ \text{Fe}^{(III)} \]

\[ \text{Enzyme} \]

\[ \text{H}_2\text{O} \]

\[ \text{Fe}^{(III)} \]

\[ \text{Enzyme} \]

Apigenin  

\[ \text{Fe}^{(IV)} \]

\[ \text{Enzyme} \]
Figure 8:

a) (2S)-Naringenin

b) (2S)-Naringenin