Mechanistic Studies on Three 2-Oxoglutarate-dependent Oxygenases of Flavonoid Biosynthesis

ANTHOCYANIDIN SYNTHASE, FLAVONOL SYNTHASE, AND FLAVANONE 3β-HYDROXYLASE

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Jonathan J. Turnbull‡, Jun-ichiro Nakajima¶, Richard W. D. Welford§, Mami Yamazaki†, Kazuki Saito¶, and Christopher J. Schofield**

From the Dyson Perrins Laboratory and The Oxford Centre for Molecular Sciences, South Parks Road, Oxford OX1 3QY, United Kingdom and the Department of Molecular Biology and Biotechnology, Graduate School of Pharmaceutical Sciences, Chiba University, Yayoi-cho 1-33, Inage-ku, Chiba 263-8522, Japan

Anthocyanidin synthase (ANS), flavonol synthase (FLS), and flavanone 3β-hydroxylase (FHT) are involved in the biosynthesis of flavonoids in plants and are all members of the family of 2-oxoglutarate- and ferrous iron-dependent oxygenases. ANS, FLS, and FHT are closely related by sequence and catalyze oxidation of the flavonoid “C ring”; they have been shown to have overlapping substrate and product selectivities. In the initial steps of catalysis, 2-oxoglutarate and dioxygen are thought to react at the ferrous iron center producing succinate, carbon dioxide, and a reactive ferryl intermediate, the latter of which can then affect oxidation of the flavonoid substrate. Here we describe work on ANS, FLS, and FHT utilizing several different substrates carried out in \( \text{O}_2/\text{H}_2\text{O} \), \( \text{O}_2/\text{H}_2\text{O}_2 \), and \( \text{O}_2/\text{H}_2\text{O}_2 \) atmospheres. In the \( \text{O}_2/\text{H}_2\text{O}_2 \) atmosphere close to complete incorporation of a single \( \text{O} \) label was observed in the dihydroflavonol products (e.g. \( 2R,3R\text{-trans-dihydrokaempferol} \)) from incubations of flavanones (e.g. \( 2S\text{-narigenin} \)) with FHT, ANS, and FLS. This and other evidence supports the intermediacy of a reactive oxidizing species, the oxygen of which does not exchange with that of water. In the case of products formed by oxidation of flavonoid substrates with a C-3 hydroxyl group (e.g. \( 2R,3R\text{-trans-dihydroquercetin} \)), the results imply that oxygen exchange can occur at a stage subsequent to initial oxidation of the C-ring, probably via an enzyme-bound C-3 ketone/3,3-\( \text{gem-diol} \) intermediate.

The flavonoids are a large class of plant secondary metabolites. They contain a 15-carbon phenylpropanoid core, which is extensively modified by rearrangement, alkylation, oxidation, and glycosylation (1). In plants the flavonoids fulfill a diverse array of roles including pigmentation and protection against UV photodamage and can act as signaling molecules (1, 2).

1 Flavonoid classes are named according to the oxidation state of the “C” ring as flavones, flavanonines, flavones, etc. Each may have different hydroxylation patterns on the “A”/“B” rings. The enzymes of the biosynthetic pathway accept a variety of A/B ring hydroxylation patterns. Where a class name is used, an example of a compound belonging to that class follows. For clarity in the figures the A and B rings are not always drawn. Use of Cahn-Ingold-Prelog nomenclature for the assignment of the absolute stereochemistry at the C-3 position of the C ring can be confusing (especially for prochiral C-3-\( \text{gem-diols} \)) due to the “priority changes” in different classes of flavonoid. Hence in the text, the faces of the flavonoid C ring are differentiated by \( \alpha \) or \( \beta \). When the A ring is drawn on the left with C-4 oxygen bond pointing down the page, the face above the plane of the paper is defined as \( \beta \), while the face beneath the plane of the paper is \( \alpha \).

2 The abbreviations used are: LCD, leucocyanidin; 2OG, 2-oxoglutarate; FHT, flavanone 3β-hydroxylase; ANS, anthocyanidin synthase; FNS, flavone synthase; FLS, flavonol synthase; DHQ, dihydroquercetin; DHK, dihydrokaempferol; HPLC, high pressure liquid chromatography; LCMS, liquid chromatography-mass spectrometry; MES, 2-(N-morpholino)ethanesulfonic acid; MOPSO, 3-(N-morpholino)-3-hydroxy-propanesulfonic acid; Tricine, N-\( \text{[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine} \).
glutarate (2OG) 7-dependent oxygenase family as are flavone synthase (FNS I), which catalyzes desaturation of (2S)-flavonones (e.g. 4) (23–26), and flavonol synthase (FLS), which catalyzes desaturation of (2R,3R)-trans-dihydroflavonols (e.g. (2R,3R)-trans-dihydroquercetin (DHQ) 8) to give flavonols (e.g. quercetin 9) (27–29). Another 2OG 7-dependent oxygenase, flavonol 6-hydroxylase, has been shown to catalyze oxidation of the flavonoid B ring (30). Oxidation of the A and B rings and desaturation of flavanones (e.g. 4) is also catalyzed by P450 heme-oxygenases (7, 8, 31–35).

The 2OG 7-dependent oxygenases (Fig. 2) are involved in a range of important pathways, including those leading to collagen, the β-lactam antibiotics, and modified amino acids and peptides (36). Roles for 2OG oxygenases have also been identified in the hypoxic signaling pathway and in DNA repair (37–41). The available evidence suggests that catalysis by 2OG oxygenases proceeds via bidentate binding of 2OG to the active site iron (42, 43). Substrate binding is thought to enable dioxygen to displace a ligating water from the catalytic iron center (44, 45). Oxidative decarboxylation of 2OG then occurs, producing succinate 10, CO₂, and a ferryl (Fe⁴⁺)=O ⇄ Fe(III)=O 11 intermediate, which can subsequently affect hydroxylation or desaturation of the substrate (Fig. 2) (46, 47).

Recent crystallographic work has demonstrated that ANS contains the double-stranded helix common to other 2OG oxygenases and identified the residues involved in substrate binding (38, 48).

ANS and FLS are closely related (50–60% sequence similarity at the polypeptide level) but display a lower level of similarity to FHT and FNS I (35%) (26, 29). This has led to the proposal that there are two distinct subgroups of flavonoid 2OG oxygenases, one containing FNS I and FHT and another containing FLS and ANS. Substrate analogue work supports this proposal; FNS I and FHT appear to possess relatively narrow substrate selectivities compared with ANS and FLS (21, 26, 28, 29, 49).

Recent in vitro work has verified the proposed role of FLS in catalyzing the conversion of (2R,3R)-trans-dihydroflavonols (e.g. 8) to flavonols (e.g. 9) (Fig. 3A) (28, 29). However, work on ANS has cast doubt on the proposal that it directly mediates oxidation of the (2R,3S,4S)-cis-leucoanthocyanidins (e.g. 6) to the pyrillium ion of the anthocyanidins (e.g. cyanidin 12) (21). In vitro, ANS (from Arabidopsis thaliana) catalyzes the four-electron oxidation of (2R,3S,4S)-cis-LEC 6 to give quercetin 9 as the predominant product (>85%) with only a trace amount (~2%) of the anticipated two-electron oxidation product, cyan...
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EXPERIMENTAL PROCEDURES

Materials—Chemicals were purchased from Sigma and were of at least analytical or molecular biology grade except for the following: organic solvents (Rathburn and Riedel-de-Haen); gases (BOC Gases); isopropyl-β-D-thiogalactoside, and dithiothreitol (Melford Laboratories Ltd.); molecular weight markers (BDH Ltd.); Bacto-tryptone, yeast extract (Difco and Oxoid Ltd.); agarose and acrylamide/bisacrylamide stock (Anachem); competent cells (Stratagene); commercial DHQ 65.6:1 for all enantiomers present.

For commercial DHQ the ratio of cistrans DHQ is 1:9 with all enantiomers present.

Table I

| Substrate          | Specific activity of 14CO2 formation from 1-[14C]2OG | Specific activity of 14CO2 formation from 1-[14C]2OG minus uncoupled turnover | Specific activity of 14CO2 formation from 1-[14C]2OG minus uncoupled turnover normalized (percentage of [2R,3S,4S]-cis-LCD with ANS) |
|--------------------|-----------------------------------------------------|-----------------------------------------------------------------------------|-----------------------------------------------------------------------------------------------------------------|
| (2R,3R)-trans-DHQ 8 | 88.6 ± 18.0                                         | 45.6                                                                       | 33                                                                                                               |
| Commercial DHQ     | 65.6 ± 13.2                                         | 22.6                                                                       | 16                                                                                                               |
| Racemic cis-DHQ     | 53.4 ± 17.3                                         | 10.4                                                                       | 8                                                                                                                |
| Racemic naringenin 4/20 | 213 ± 53.2                                   | 170                                                                       | 123                                                                                                              |
| (2S)-Naringenin 4   | 231 ± 19.8                                         | 188                                                                       | 136                                                                                                              |
| (2R)-Naringenin 20  | 157 ± 15.8                                         | 114                                                                       | 83                                                                                                                |
| Racemic cis-LCD     | 84.1 ± 6.9                                         | 41                                                                         | 30                                                                                                                |
| (2R,3S,4S)-cis-LCD 6 | 181.5 ± 28.7                                | 138                                                                       | 100                                                                                                               |
| Racemic trans-LCD   | 188.4 ± 19.3                                       | 145                                                                       | 105                                                                                                               |
| (2R,3S,4R)-trans-LCD 13 | 385.0 ± 31.4                                | 342                                                                       | 248                                                                                                               |

Table II

| Substrate          | Specific activity of 14CO2 formation from 1-[14C]2OG | Specific activity of 14CO2 formation from 1-[14C]2OG minus uncoupled turnover | Specific activity of 14CO2 formation from 1-[14C]2OG minus uncoupled turnover normalized (percentage of [2R,3S,4S]-cis-LCD with ANS) |
|--------------------|-----------------------------------------------------|-----------------------------------------------------------------------------|-----------------------------------------------------------------------------------------------------------------|
| (2R,3R)-trans-DHQ 8 | 148.0 ± 87                                         | 81.5                                                                       | 59                                                                                                               |
| Commercial DHQ     | 110.6 ± 18.1                                        | 44.1                                                                       | 32                                                                                                               |
| Racemic cis-DHQ     | 61.7 ± 10.2                                         | 0                                                                         | 0                                                                                                                |
| Racemic naringenin 4/20 | 460.0 ± 87.5                                | 393.5                                                                       | 285                                                                                                              |
| (2S)-Naringenin 4   | 447.2 ± 15.05                                       | 380.7                                                                       | 276                                                                                                              |
| (2R)-Naringenin 20  | 434.0 ± 31.3                                       | 367.5                                                                       | 266                                                                                                              |
| (2R,3S,4S)-cis-LCD 6 | 53.9 ± 4.0                                     | 0                                                                         | 0                                                                                                                |
| (2R,3S,4R)-trans-LCD 13 | 128.4 ± 10.4                                | 61.9                                                                       | 49                                                                                                               |

FIG. 2. General reaction scheme for 2OG, non-heme Fe(II)-dependent oxygenases. Enz, enzyme.

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idin 12, being observed (Fig. 3B) (50). Incubations of (2R,3S,4R)-trans-LCD 13 with ANS gave cis-DHQ 14, trans-DHQ 8/15, quercetin 9, and cyanidin 12 (Fig. 3C) (21, 49, 50). ANS also catalyzes the natural reaction of FLS, i.e., conversion of (2R,3R)-trans-DHQ 8 to quercetin 9 (Fig. 3A) (21). Both ANS and FLS have also been found to react with the unnatural substrate naringenin 4 (Fig. 3, D and E) (26, 28, 29, 49). These and other results led to the proposal that ANS mediates oxidation of the natural (2R,3S,4S)-cis-LCD 6 substrate via initial oxidation at C-3 followed by loss of water from the 3,3-gem-diol 10 to give a C-2,C-3 enol (a (4S)-flav-2-en-3,4-diol 17). In vitro, this may be (partially) retained in the active site to undergo a further round of oxidation to give quercetin 9. It has been proposed that in vivo the (4S)-flav-2-en-3,4-diol 17 may be directly channeled to flavonoid glycosyltransferase, which is responsible for cyanin 5 formation (22, 50) (Fig. 1).

Here we report work that enhances mechanistic understanding of the non-heme dioxygenases of flavonoid biosynthesis by carrying out assays of ANS, FHT, and FLS in 18O-labeled dioxygen and water with a variety of substrates.
β,β-thiogalactoside when the A600 reached 0.6–0.8. Growth continued at 37 °C for 3.5 h before harvesting the cells and storage at −80 °C until further use. A clear lysate containing the recombinant A. thaliana FLS was prepared in the same manner as for A. thaliana ANS. The soluble portion of the cell lysate was applied to a Q-Sepharose FF column pre-equilibrated at 50 mM Tricine, pH 7.3 before elution using a linear gradient of NaCl from 0 to 0.5 M NaCl over 5 column volumes. FLS eluted at 230 mM NaCl, and the fractions containing FLS at 45–55% purity (by SDS-PAGE analysis) were pooled and further purified. The FLS protein was concentrated and buffer-exchanged into 50 mM MES, pH 6.15, 10% (w/v) glycerol before loading onto the reactive green C-19 column equilibrated using the same buffer. The protein was eluted with a linear gradient of NaCl from 0 to 2M NaCl. Fractions of 90–95% purity were pooled, concentrated, buffer-exchanged, and then stored at −80 °C. Petunia hybrida ANS was prepared as reported previously (22). P. hybrida FHT and FLS were prepared from corresponding cDNAs amplified by PCR as for P. hybrida ANS.

18O Labeling Experiments—For experiments in 18O2/16OH2, 16O2/18OH2, and 18O2/18OH2 environments, reagent solutions were initially...
mixed under an argon atmosphere (<0.4–0.8 ppm O₂) in a Belle Technology® glove box to a total volume of 500, 70, and 70 μl, respectively. The FeSO₄·7H₂O, ascorbate, 20G, substrates (except the unstable leucoanthocyanidins), 5-mL tubes, solid reagents, and rubber septa were ported into a glove box 24 h before use. The leucoanthocyanidin substrates (dissolved in Milli-Q H₂O) and purified ANS and FLS were ported into the glove box immediately prior to use. H₂O used for assays in ¹⁸O/H₂O and ¹⁸O/H₂O environments was 95% ¹⁸O-labeled. The MOPSO buffer used was prepared by addition of 1 M MOPSO, pH 6.2 to the 95% ¹⁸O-labeled H₂O. The assay reagents were all dissolved in either Milli-Q H₂O or MeOH. This lead to a ~80% ¹⁸O-labeled aqueous environment. The reagents were mixed in the following order to give final concentrations of 50 mM MOPSO, pH 6.2, 40 mM ascorbate, 20 mM 2OG, 40 μM FeSO₄·7H₂O, 0.2 mg ml⁻¹ enzyme, 800 μM substrate (MeOH).

HPLC/MS Assays—HPLC/MS was carried out using a Synergi C-18 MAX column (250 mm × 4.6 mm; bead size, 4 μm; Phenomenex). Analyses utilized a photodiode array detector and a Micromass ZMD mass spectrometer (electrospray ionization). All HPLC grade buffers were filtered through 0.2-μm filters and sparged with helium(g) at 100 ml min⁻¹ for 20 min before use. Buffers were acidified using formic acid, 2% (v/v) for HPLC and 0.05% (v/v) for LCMS buffers. Prior to injection 1% (v/v) formic acid was added to samples, and they were centrifuged at 13,000 rpm for 10 min. After injection samples were eluted isocratically for 10 min in 10% MeOH, and then a gradient was run from 10 to 80% MeOH over 25 min followed by 10 min of eluting isocratically with 80% MeOH.

Substrate Synthesis—(2R,3S,4R)-trans-LCD 13 and (2R,3S,4S)-cis-LCD 6 were synthesized and purified as reported previously (50, 53). (2R)- and (2S)-naringenin 4 were produced by naringinase-mediated cleavage of the glycosyl bond of (2R)- and (2S)-naringin, respectively (30 °C, pH 4.0, 100 mM NaH₂PO₄ buffer for 30 min). (2R)- and (2S)-naringenin were extracted from the reaction mixtures by solid phase extraction. Strata C18 solid phase extraction columns (3-mL, 500-mg sorbent bed) from Phenomenex were mounted onto an IST Vacmaster manifold, and solvent was washed through the columns in vacuo. All columns were primed with MeOH (4 mL) and equilibrated with H₂O (4 mL) before the sample was loaded. The column was washed sequentially with 4-mL aliquots of 20 and 40% MeOH (v/v) before elution of the purified (2R)- and (2S)-naringenin with 6 mL of 100% MeOH (v/v). The MeOH was removed in vacuo, and the water was removed by lyophilization.

A racemic mixture of cis/trans-DHQ was produced by C-2 epimerization of commercial DHQ as reported previously (54). cis-DHQ was purified from the cis/trans-DHQ mixture as follows. Racemic cis/trans-DHQ was dissolved in MeOH (10 mg, 1 mL) and loaded onto a Phenomenex Luna C-18 250-mm × 10-mm (bead size, 4 μm) column pre-equilibrated with 50 mM NaH₂PO₄, pH 6.8, 20% MeOH (v/v) and run at 3.6 mL min⁻¹. The column was run isocratically for 15 min in 50 mM NaH₂PO₄, pH 6.8, 20% MeOH (v/v) before a gradient was run to 50 mM NaH₂PO₄, pH 6.8, 50% MeOH (v/v) over 25 min followed by 15 min of isocratic elution. The peaks corresponding to the racemic DHQ diastereomers were collected, diluted to <10% MeOH by addition of Milli-Q H₂O, and freeze-dried. The purified DHQ (trans- and cis-) diastereomers were dissolved in the minimum volume of 50 mM NaH₂PO₄, pH 6.8, 20% MeOH (v/v) and then loaded onto Strata C18 solid phase extraction columns pre-equilibrated as for the purification of the naringinase reaction mixture above. The columns were washed with 10 mL of Milli-Q H₂O before elution of DHQ in 6 mL of 100% MeOH (v/v). The presence of the DHQ isomers was confirmed by UV analysis of the eluent before evaporation of the MeOH at 30 °C in vacuo and further drying on a high vacuum line. NMR analysis in MeOH-OD₄ confirmed the identity of the purified racemic cis-DHQ containing <10% trans-DHQ. Selected ¹H NMR data (54): trans-DHQ δ₁ (500 MHz): 4.6 (d, 1H, J 11.5 Hz, H-3), 5.0 (d, 1H, J 11.5 Hz, H-2), 5.8–6.0 (2ca d, 1H, J 2.0 Hz, H-6, H-8), 6.7–7.0 (m, 3H, H-2’, H-3’, H-4’); cis-DHQ δ₂ (500 MHz): 4.2 (d, 1H, J 3.0 Hz, H-3), 5.3 (d, 1H, J 3.0 Hz, H-2), 5.8–6.0 (2ca d, 1H, J 2.0 Hz, H-6, H-8), 6.7–7.0 (m, 3H, H-2’, H-3’, H-4’).

RESULTS AND DISCUSSION

The Effect of Different Flavonoid Substrates on the Rate of ANS- and FLS-catalyzed Oxidative Decarboxylation of 2OG—Prior to the labeling studies we assessed the activities of ANS and FLS (both A. thaliana) in the presence of a variety of natural and unnatural substrates by measuring release of ¹³CO₂ from 1-[¹³C]2OG 7 (Fig. 2 and Tables I and II). While it is known that prime substrate oxidation is not always fully coupled to that of 2OG 7 (4, 55–58), the results support previous work implying that both ANS and FLS possess a broad substrate selectivity in vitro (21, 26, 28, 29, 49) and provide some new insights.

Incubation of LCDs with ANS and FLS—ANS (A. thaliana) catalyzes the in vitro oxidative decarboxylation of 2OG at a greater rate in the presence of (2R,3S,4R)-trans-LCD 13 than with its natural substrate, (2R,3S,4S)-cis-LCD 6 (Table I) (50). As described above, it has been suggested that in vitro the initial product of oxidation of (2R,3S,4S)-cis-LCD 6, a (4S)-flav-2-en-3,4-diol 17, does not efficiently leave the active site of ANS; instead it is proposed to undergo a further round of oxidation giving quercetin 9. This “trapping” of the (4S)-flav-2-en-3,4-diol 17 in the active site may explain the reduced rate of 2OG 7 oxidation by ANS with the (2R,3S,4S)-cis-LCD 6 substrate compared with (2R,3S,4R)-trans-LCD 13. Although both (2R,3S,4S)-cis-LCD 6 and (2R,3S,4R)-trans-LCD 13 are substrates for ANS, the 2OG 7 turnover and HPLC analyses (data not shown) indicate that only the trans-isomer 13 is a substrate for FLS; FLS may have evolved to deselect the ANS substrate. ANS showed a preference for the natural C-2,C-3 stereochemistry, i.e. (2R,3S,4S)-cis- 6 and (2R,3S,4R)-trans-LCD 13 compared with racemic mixtures of these substrates (Table I).

Incubation of DHQ Stereosomers with ANS and FLS—With DHQ as a substrate, both ANS and FLS (both A. thaliana) showed a preference for the “natural” (2R,3R)-trans 8 stereochemistry (Tables I and II). Both 2OG 7 turnover and HPLC assays imply that racemic cis-DHQ is a poor substrate for ANS.
and FLS (HPLC data not shown). This is probably due to the unnatural C-2 stereochemistry of (2'S,3'R)-cis-DHQ and/or complexion of the (3'S)-hydroxyl of (2'R,3'S)-cis-DHQ 14 to the active site iron, preventing dioxygen binding. Recently Martens et al. (26) showed that (2'R,3'S)-cis-dihydrokaempferol (DHK) 18 is a very poor substrate for FLS (Petroselinum crispum) but is an efficient substrate for FNS I. The trans-dihydroflavonols (e.g. (2'R,3'R)-trans-DHK 19 and (2'R,3'R)-trans-DHQ 8) are the natural substrates of FLS (28, 29) and are also efficient substrates of ANS (21); however, they are not accepted as sub-

### Table III

| Enzyme | Species | Assay conditions | Substrate | Percentage of 18O incorporation in dihydrokaempferol product(s) |
|--------|---------|-----------------|-----------|---------------------------------------------------------------|
| ANS    | A. thaliana | 18O2/16OH2 | Racemic naringenin 4/20 | 93 % |
| ANS    | A. thaliana | 18O2/16OH2 | (2S)-Naringenin 4 | 86 % |
| ANS    | A. thaliana | 18O2/16OH2 | (2R)-Naringenin 20 | 85 % |
| FLS    | A. thaliana | 18O2/16OH2 | Racemic naringenin 4/20 | 96 % |
| FLS    | A. thaliana | 18O2/16OH2 | (2S)-Naringenin 4 | 89 % |
| FLS    | A. thaliana | 18O2/16OH2 | (2R)-Naringenin 20 | 96 % |
| FHT    | P. hybrida | 18O2/16OH2 | (2S)-Naringenin 4 | 93 % |
| ANS    | A. thaliana | 16O2/16OH2 | Racemic naringenin 4/20 | 0 % |
| FLS    | A. thaliana | 16O2/16OH2 | Racemic naringenin 4/20 | 8 % |
| FHT    | P. hybrida | 16O2/16OH2 | (2S)-Naringenin 4 | 0 % |

In some cases the apparent low levels of 18O incorporation in an 18O2 atmosphere may in part be due to a failure to completely remove 16O2 from the sample. The exact site of 18O incorporation was not determined.

### Table IV

The percentage of incorporation of 18O label in the quercetin 9 product of reactions of ANS and FLS with DHQ substrates

For commercial DHQ the ratio of cis/trans DHQ is 1:9 with all enantiomers present. The exact site of 18O incorporation was not determined.

| Enzyme | Species | Assay conditions | Percentage of 18O incorporation in the quercetin 9 product |
|--------|---------|-----------------|------------------------------------------------------------|
| ANS    | A. thaliana | 18O2/16OH2 | (2R,3R)-trans-DHQ 8 | 3 % |
| ANS    | A. thaliana | 18O2/16OH2 | Commercial DHQ | 2 % |
| ANS    | A. thaliana | 18O2/16OH2 | Racemic cis-DHQ | 0 % |
| FLS    | A. thaliana | 18O2/16OH2 | (2R,3R)-trans-DHQ 8 | 3 % |
| FLS    | A. thaliana | 18O2/16OH2 | Commercial DHQ | 4 % |
| FLS    | A. thaliana | 18O2/16OH2 | Racemic cis-DHQ | 10 % |
| FLS    | A. thaliana | 18O2/16OH2 | Commercial DHQ | 0 % |
| ANS    | A. thaliana | 18O2/16OH2 | (2R,3R)-trans-DHQ 8 | 71 % |
| ANS    | A. thaliana | 18O2/16OH2 | Commercial DHQ | 65 % |
| ANS    | A. thaliana | 18O2/16OH2 | Racemic cis-DHQ | 88 % |
| FLS    | A. thaliana | 18O2/16OH2 | (2R,3R)-trans-DHQ 8 | 76 % |
| FLS    | A. thaliana | 18O2/16OH2 | Commercial DHQ | 76 % |
| FLS    | A. thaliana | 18O2/16OH2 | Racemic cis-DHQ | ND * |
| FLS    | A. thaliana | 18O2/16OH2 | Commercial DHQ | 52 % |

* Not determined.

### Table V

The percentage of incorporation of 18O label into the products of ANS reactions with leucoanthocyanidin substrates

The absolute chirality of the cis- and trans-DHQ and the exact site of 18O incorporation were not determined.

| Enzyme | Species | Assay conditions | Substrate Product(s) | Percentage of 18O incorporation |
|--------|---------|-----------------|----------------------|-------------------------------|
| ANS    | A. thaliana | 18O2/16OH2 | Commercial LPD * | DHK 2 % |
| ANS    | A. thaliana | 18O2/16OH2 | Commercial LCD | trans-DHQ 7 % |
| ANS    | A. thaliana | 18O2/16OH2 | (2R,3S,4S)-cis-LCD 6 | cis-DHQ 4 % |
| ANS    | A. thaliana | 18O2/16OH2 | (2R,3S,4R)-trans-LCD 13 | Quercetin 9 3 % |
| ANS    | A. thaliana | 18O2/16OH2 | Commercial LCD | cis-DHQ 5 % |
| ANS    | A. thaliana | 18O2/16OH2 | cis-DHQ 4 % |
| ANS    | A. thaliana | 18O2/16OH2 | Quercetin 9 3 % |
| ANS    | A. thaliana | 18O2/16OH2 | Quercetin 9 0 % |
| ANS    | A. thaliana | 18O2/16OH2 | Quercetin 9 0 % |
| ANS    | A. thaliana | 18O2/16OH2 | Quercetin 9 0 % |
| ANS    | A. thaliana | 18O2/16OH2 | Quercetin 9 100 % |
| ANS    | A. thaliana | 18O2/16OH2 | trans-DHQ 63 % |
| ANS    | A. thaliana | 18O2/16OH2 | cis-DHQ 78 % |
| ANS    | A. thaliana | 18O2/16OH2 | Quercetin 9 69 % |
| ANS    | A. thaliana | 18O2/16OH2 | Quercetin 9 53 % |
| ANS    | A. thaliana | 18O2/16OH2 | Quercetin 9 93 % |
| ANS    | A. thaliana | 18O2/16OH2 | Cyanidin 12 100 % |

* Leucopelargonidin.
strates by FNS I (24). These results together demonstrate a clear difference in the substrate selectivities of the ANS/FLS and FNS I/FHT subfamilies.

**Incubation of Naringenin Enantiomers with ANS and FLS—**
Incubation of $(2R)$- and $(2S)$-naringenin 4 with ANS or FLS (A. thaliana) demonstrated comparable rates of 2OG decarboxylation in the presence of both enantiomers. $(2S)$-naringenin 4 is an *in vivo* substrate for FNS I (Tables I and II). This observation contrasts with the results for the different enantiomers of the trans-DHQ 8/15 and LCD substrates (e.g., the activity for pure $(2R,3S,4S)$-cis-LCD 6 was twice that of racemic cis-LCD) where a preference for the natural 2,3-trans stereochemistry was observed. The presence of a C-3 hydroxyl group (absent in naringenin 4/20) is thus important in biasing the substrate selectivity of ANS and FLS toward the natural C-2 stereochemistry (equivalent to $(2R,3R)$ for DHQ).

Support for this conclusion comes from the observation that incubation of $(2R)$-naringenin 20 with FLS (A. thaliana or Citrus unshiu)
The C-3 hydroxylation of naringenin—by initial abstraction of the possible mechanisms for FNS I action (Fig. 4), both proceeding of an ordered sequential mechanism in which dehydration can dehydration. However, this evidence does not rule out the possibility e.g. 18 FHT (both and unnatural substrates by these enzymes occurs via initial by ANS, FLS, and FHT suggests that oxidation of both natural 4 S of its natural substrate (2 S)-naringenin 4-ate/product was not detected during FNS I-mediated oxidation work showed that an enzyme-free C-3-hydroxylated intermedi- rate of (2 S)-DHK 8, can give the quercetin 9 product. Hypothesis that FNS I is a C-3 oxygenase. However, recent shows that an enzyme-free C-3-hydroxylated intermedi- ate/product was not detected during FNS I-mediated oxidation of its natural substrate (2 S)-naringenin 4 (26). Further incubation of a C-3-hydroxylated flavanone (i.e. a dihydroflavonol, e.g. 18) with FNS I did not result in enzyme-mediated dehy- dration. However, this evidence does not rule out the possibility of an ordered sequential mechanism in which dehydration can only occur subsequent to formation of a dihydroflavonol intermediate in the active site. Consistent with this, we propose two possible mechanisms for FNS I action (Fig. 4), both proceeding by initial abstraction of the β-face C-3 hydrogen. There are then two possibilities, either a non-concerted divinicial C-2,C-3 desaturation (path A) or a sequential β-face hydroxylation and dehydration (path B).

The stereochemistry of the C-3 hydroxylation products of FLS (and ANS)3 (both A. thaliana) with naringenin substrates shows that α-face hydroxylation predominates, i.e. incubation of (2S)-naringenin 4 leads to (2R,3S)-cis-DHK 18 (Fig. 3D) and (2R)-naringenin 20 leads to (2S,3S)-trans-DHK 21 (Fig. 3E). In contrast, FHT is a β-face hydroxylase, i.e. (2S)-naringenin 4 leads to (2R,3R)-trans-DHK 19 (Fig. 1). Thus, the two subfamilies of 2OG oxygenases in flavonoid biosynthesis can be further classified into C-3 α-face oxygenases (ANS/FLS) and C-3 β-face oxygenases (FHT/FNS I). Support for this proposal comes from the observation that unnatural substrates of ANS/FLS, which are accepted efficiently (Tables I and II), have a C-3 α-face hydrogen. Further to this, FNS I converts (2R,3S)-cis-DHK 18 (in which, like its natural substrate (2S)-naringenin 4, both C-2 and C-3 hydrogens are on the β-face) but does not convert

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Fig. 7. Dehydration of 3,3-gem-diol 24 or tautomeration of 3-one 25, both formed by ANS/FLS-catalyzed C-3 oxidation of (2R,3R)-trans-DHK 8, can give the quercetin 9 product. leads to products including (2S,3S)-trans-DHK 21, a compound with “unnatural” C-2 and C-3 stereoisomerism (28, 29) (Fig. 3E). In comparison, FNS I (P. crispum) and FHT (P. hybrida) demonstrate a high C-2 selectivity with (2R)-naringenin 20 not being accepted as a substrate (24, 29). This suggests that the ANS/FLS subfamily may select for substrates with a β-face C-3 hydroxyl group. In comparison, the presence of the C-2 α-face B ring is relatively more important for FHT/FNS I. The higher C-2 stereoselectivity of FHT/FNS I is perhaps unsurprising as unlike ANS/FLS their natural substrates (2S)-flavanones (e.g. 4) do not possess a C-3 hydroxyl group.

Differential Substrate Selectivities of ANS/FLS and FHT/ FNS I—The C-3 hydroxylation of naringenin 4/20 substrates by ANS, FLS, and FHT suggests that oxidation of both natural and unnatural substrates by these enzymes occurs via initial C-3 oxidation. The high sequence similarity between FNS I and FHT (both P. crispum) (94% sequence homology) also supports the hypothesis that FNS I is a C-3 oxygenase. However, recent work showed that an enzyme-free C-3-hydroxylated intermedi- ate/product was not detected during FNS I-mediated oxidation of its natural substrate (2S)-naringenin 4 (26). Further incubation of a C-3-hydroxylated flavanone (i.e. a dihydroflavonol, e.g. 18) with FNS I did not result in enzyme-mediated dehy- dration. However, this evidence does not rule out the possibility of an ordered sequential mechanism in which dehydration can only occur subsequent to formation of a dihydroflavonol intermediate in the active site. Consistent with this, we propose two possible mechanisms for FNS I action (Fig. 4), both proceeding by initial abstraction of the β-face C-3 hydrogen. There are then two possibilities, either a non-concerted divinicial C-2,C-3 desaturation (path A) or a sequential β-face hydroxylation and dehydration (path B).

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mammalian type I prolyl-4-hydroxylase (62), and hypoxic inducible factor hydroxylases (63, 64), show that >90% input of oxygen from dioxygen occurs on hydroxylation of their substrates. This contrasts with results for eukaryotic lysyl hydroxylase where <10% of 18O was incorporated into the product (65) (data reviewed in Mehn et al. (66)).

Assays with ANS, FLS, and FHT were carried out in labeled oxygen environments of 18O2/16OH2, 16O2/18OH2, and 18O2/18OH2 with a variety of substrates. To ensure that the results were representative, experiments were carried out with ANS and FLS from A. thaliana and P. hybrida. Incubations were also carried out with FHT from P. hybrida. Product formation and incorporation of the label was measured using LCMS. Comparisons of the 18O incorporation in assays with ANS, FLS, and FHT (Tables III, IV, and V) revealed several trends, implying certain common mechanistic processes.

(i) C-3 hydroxylation of naringenin 4/20 by ANS and FLS (both A. thaliana) and FHT (P. hybrida) revealed high levels of 18O incorporation from incubation in an 18O2 atmosphere (Table III). Only very low levels of 18O incorporation were observed in incubations in a 16O2/18OH2 environment. This indicates that the oxygen of the introduced C-3 hydroxyl group is derived from dioxygen (Fig. 5), and at least in the case of naringenin 4/20 oxidation, the ferryl oxygen 11 (or other intermediate) does not readily exchange with water during catalysis.

(ii) On incubation of ANS/FLS (A. thaliana or P. hybrida) with trans-DHQ 8/15 or LCD 6/13 substrates under an 18O2/16OH2 atmosphere no 18O label was incorporated into the products (Tables IV and V). If, as previously proposed, C-3 oxidation is the initial step in catalysis (48, 49), together with (i), this is consistent with a mechanism in which the initially introduced hydroxyl group is subsequently lost.

(iii) Incubation of ANS/FLS (A. thaliana or P. hybrida) with trans-DHQ 8/15 or LCD 6/13 substrates in 18OH2 environments led to some 18O incorporation into enzymatic products. Control experiments implied that the incorporation was not into the phenolic OH groups of the products and that non-enzymatic oxygen exchange does not occur at either the oxygen of the C-3 hydroxyl group or C-4 ketone of the quercetin 9 product on the time scale of the incubation. Together with (i) and (ii), these observations imply that introduction of the 18O label from 18OH2 occurs at an intermediate stage following initial substrate oxidation.

Since 2OG oxygenases commonly catalyze hydroxylations and both ANS and FLS can catalyze the C-3 hydroxylation of naringenin 4/20, it seems likely that ANS and FLS react with leucoanthocyanidin (e.g. 6) and dihydroflavonol (e.g. 8) substrates by initial C-3 oxidation. Two pieces of evidence suggest that, as with the eukaryotic oxygenases, the ferryl 11 (or other reactive oxygen species) does not exchange during catalysis by the flavonoid oxygenases ANS, FLS, and FHT: first, the high level of incorporation of 18O from 18O2 gas into the products of incubations with naringenin 4/20 (Table III), and second, the observation that where exchange does occur, different levels of incorporation are seen in 18O2/16OH2 and 18O2/18OH2 environments (Tables IV and V). Equilibration between 3-one 22 or 3,3-gem-diol 23 intermediates provides a mechanism of oxygen exchange after initial oxidation, rationalizing the incorporation of some 18O label from 18OH2 into products derived from substrates with a C-3 hydroxyl group (Fig. 6). For oxidation of trans-DHQ 8/15 and leucoanthocyanidin (e.g. 6) substrates in a 18O2/16OH2 atmosphere by ANS/FLS, the lack of 18O label in the products obtained is consistent with initial C-3 hydroxylation, providing the introduced C-3 hydroxyl is stereospecifically removed in a subsequent step. Since both (2R,3S,4S)-cis-LCD 6 and (2R,3R)-trans-DHQ 8 possess a β-face hydroxyl group, the β-face C-3 hydroxyl of the 3,3-gem-
diol 23 intermediate will be derived from the substrate, while the α-face hydroxyl will be derived from dioxygen (assuming no inversion at C-3) (Fig. 6). Four pieces of evidence suggest that the α-face hydroxyl group might be coordinated, or in close proximity, to the active site iron. (i) Structural work implies that the C-3 α-face hydroxyl of a 3,3-gem-diol 23 is likely to lie in close proximity to the active site iron, which may function as a Lewis acid promoting its stereospecific removal (50). (ii) The observation of ANS/FLS with (2S)-naringenin 4 produces predominantly (2R,3S)-cis-DHK 18 (i.e. α-face C-3 hydroxyl) (26, 29, 49). (iv) The formation of the thermodynamically less stable (2R,3S)-cis-DHQ 14 diastereoisomer in incubations with (2R,3S)-trans-LCD 13 substrates suggests that the active site is orientated so that complexation of an α-face hydroxyl group to the active site Fe(II) is favored (21, 49).

Coordination of the α-face hydroxyl group to the active site iron, which could act as a Lewis acid, will favor its stereospecific removal (Fig. 6). In an 18O2/16OH2 environment the 3-one intermediate derived via loss of the α-face hydroxyl will have been delivered from the ferryl 11, therefore likely to be proximate to the ferryl. (iii) Incubation of ANS and FLS with (2S)-naringenin 4 reveals that the C-3 face hydroxyl will have been delivered from the ferryl 11, therefore likely to be proximate to the ferryl. (iii) Structural work implies that the C-3 α-face hydroxyl of a 3,3-gem-diol 23 is likely to lie in close proximity to the active site iron, which may function as a Lewis acid promoting its stereospecific removal (50). (ii) The presence of ANS/FLS (Tables IV and V). The observed level of oxidation product of (26, 29, 49) also reveal that the ANS/FLS and FHT/FLS pairs may be classified as α- and β-face-selective oxygenases, respectively. The 18O labeling experiments imply that this is true even when the products do not possess a chiral center at C-3, e.g. ANS-mediated production of quercetin 9 from (2R,3S,4S)-cis-LCD 6. The results also demonstrate that, even when oxygen exchange between dioxygen and water does occur, this is not via exchange of an iron-bound reactive oxidizing species but most likely via C-3 ketone 22/gem-diol 23 equilibration subsequent to the oxidation of the flavonoid C ring (Fig. 6). The lack of significant exchange of the ferryl intermediate renders ANS, FLS, and FHT similar to most but not all eukaryotic 2OG oxygenases (59–66).

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Mechanistic Studies on Three 2-Oxoglutarate-dependent Oxygenases of Flavonoid Biosynthesis: ANTHOCYANIDIN SYNTHASE, FLAVONOL SYNTHASE, AND FLAVANONE 3β-HYDROXYLASE
Jonathan J. Turnbull, Jun-ichiro Nakajima, Richard W. D. Welford, Mami Yamazaki, Kazuki Saito and Christopher J. Schofield

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