Mechanistic Study on the Oxidation of Anthocyanidin Synthase by Quantum Mechanical Calculation*

Received for publication, January 11, 2006, and in revised form, May 15, 2006. Published, JBC Papers in Press, May 15, 2006, DOI 10.1074/jbc.M600303200

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Anthocyanidin synthase (ANS), a member of the 2-oxoglutarate-dependent dioxygenase family in flavonoid biosynthesis, catalyzes the conversion of leucoanthocyanidins (e.g. 2R,3S,4S-cis-leucoanthocyanidin, LCD) to flav-2-en-3,4-diols, a direct precursor of colored anthocyanidins via flavan-3,3,4-triols. The detailed oxygenation mechanism of 2R,3S,4S-cis-LCD to flav-2-en-3,4-diols was investigated using the density functional theory method. An initial model for the calculation was constructed from a structure obtained by a 100-ps molecular dynamics simulation of Arabidopsis ANS under physiological conditions. This model consisted of an LCD molecule as the substrate together with an iron atom, two histidine residues, an aspartic acid residue, a succinate, and an oxygen atom as ligands of the iron atom. The results of the calculation indicated that both the C-3 and C-4 positions of LCD can be oxidized, although C-4 oxidation is preferable. The C-3 oxidation required several steps to form flavan-3,3,4-triol: 1) formation of Fe(H-)-OH and a substrate C-3 radical via hydrogen atom abstraction by Fe(V)=O, 2) formation of a C-3 ketone and a water molecule, 3) addition of OH into the C-3 position of the ketone, and 4) addition of H+ to form flavan-3,3,4-triol. On the other hand, C-4 oxidation of 2R,3S,4S-cis-LCD resulted in the direct formation of 2R,3-trans-dihydroquercetin. These results suggest that the oxidation at C-3 of LCD, a key reaction for coloring in anthocyanin biosynthesis, can be regarded as a “side reaction” from the viewpoint of quantum mechanics of enzymatic reactions. Molecular evolution implications of ANS and related proteins are discussed in terms of reaction dynamics.

Flavonoids are a large class of plant secondary metabolites on which extensive study has been carried out in the areas of chemistry, biochemistry, and genetics. In particular, anthocyanins are well known to be the main pigments in flowers and also play a role in protection against UV photodamage (1). The antioxidant activity of anthocyanins allows a variety of medical uses, such as the prevention of cancer, anti-inflammatory activity, anti-arteriosclerosis activity, etc. (2).

Comprehensive molecular biological and biochemical studies have already established the general biosynthesis steps that produce anthocyanin in plants, and most of the genes of the biosynthetic enzymes have been cloned and characterized (3–6). In general, the first colored compound in the biosynthetic pathway of anthocyanin is a flavylum ion form of anthocyanidins (e.g. cyanidin 1), which is derived from colorless leucoanthocyanidins (e.g. 2R,3S,4S-cis-leucoanthocyanidin 2 (LCD) (7)). Anthocyanidin synthase (ANS) is responsible for this step, i.e. dehydrogenation from C-2 and dehydration from C-3 and C-4 formally (Fig. 1A). The cDNA encoding ANS was first isolated from a Zea mays mutant (a2) by transposon tagging (7); however, no biochemical investigation succeeded until the first study using the recombinant ANS enzyme of Perilla frutescens (8). Successful in vitro studies using recombinant ANSs of Petunia hybrida, Antirrhinum majus, Z. mays, and Torenia fournieri have also been carried out, confirming that ANS is a member of the 2-oxoglutarate-dependent dioxygenase family, which requires Fe(II)+, 2-oxoglutarate, 3, and ascorbate for its oxygenation activity (Fig. 1B) (9). An important feature of the reaction catalyzed by ANS is that no additional enzyme such as dehydratase is required, despite the involvement of a formal dehydration step. The direct precursor for the flavylum ion form of anthocyanidin is presumed to be 4S-flav-2-en-3,4-diol (Fig. 1A) (9).

A recent in vitro study on ANS revealed that ANS is capable of catalyzing the oxygenation of 2R,3-trans-dihydroquercetin (DHQ) 5 toward quercetin 6; this reaction is also catalyzed by flavonol synthase (FLS), another member of the 2-oxoglutarate-dependent dioxygenase family (10). Surprisingly, in previous studies, most of the products obtained by Arabidopsis ANS from 2R,3S,4S-cis-LCD 2 were not the expected product cyanidin 1 but rather quercetin 6 and cis-7-trans-5 DHQ (10, 11) (Fig. 1A). ANS and FLS can be categorized as 3α-oxygenases, since they can also accept 2S-naringenin 8 (a natural substrate for flavanone 3β-hydroxylase) to connect to the corresponding 3α-hydroxylated compounds (2).

* This work was supported in part by grants-in-aid for Scientific Research from the Ministry of Education, Science, Sports and Culture, Japan and by CREST of JST (Japan Science and Technology). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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§ For clarity in the figures, the A and B rings are not always drawn.

¶ The abbreviations used are: LCD, leucoanthocyanidin; ANS, anthocyanidin synthase; DHQ, dihydroquercetin; FLS, flavonol synthase; FGT, flavonoid glycosyltransferase; MD, molecular dynamics; DFT, density functional theory; DFR, dihydroflavonol reductase; MES, 2-(N-morpholino)ethansulfonic acid.
derivative, 2R,3S-cis-dihydrokaempferol 9 (Fig. 1A) (12–16). Recent in vitro work using 18O stable isotope implied a possible detailed reaction mechanism of ANS, specifically, that the C-3 α-face of 2R,3S,4S-cis-LCD 2 might be hydroxylated to form 2R,4S-flav-3,3,4-triol 10 (3,3-gem-diol) and is likely to be equilibrated with 2R,4S-flav-3-en-3,4-diol 11 (3-ketone) during enzyme binding (17). Dehydration from C-2 and C-3 or from C-3 and C-4 of the 3,3-gem-diol 10 or tautomerization of 3-ketone 11 leads to the formation of 4S-flav-2-en-3,4-diol 4 or flav-3-en-3,4-diol 12, i.e. the step from the oxygenation of LCD 2 to the formation of 3,3-gem-diol 10 (Fig. 1C). The present simulation suggests that oxygenation at both the C-3 and C-4 positions of 2R,3S,4S-LCD 2 is possible, although C-4 oxidation seems to occur more easily than C-3 oxidation. The detailed reaction mechanisms are discussed below in the context of an evolutionary consequence of ANS and related proteins.

*Oxidation Mechanisms by Anthocyanidin Synthase*

![FIGURE 1. Current proposals for the flavonoid biosynthetic pathway. A, current proposals for ANS reaction. B, simplified reaction sequence of 2-oxoglutarate-dependent dioxygenases. C, pathways for the production of cyanidin 1, trans-/-cis-DHQ 5/7, and quercetin 6 during in vitro ANS catalysis. Boxes indicate the reactions investigated in this paper. CHS, chalcone synthase; CHI, chalcone isomerase; FNS, flavone synthase; F3H, flavanone 3β-hydroxylase; DHK, dihydrokaempferol.](image-url)
**MATERIALS AND METHODS**

**Molecular Dynamics Simulation**—A molecular dynamics (MD) simulation was performed to determine the appropriate structure of the ferryl (Fe(IV)/O/Fe(III)–O/H2O) species in the enzyme. The three-dimensional crystal structure of ANS-Fe(II)-succinate-quercetin complex (post-reaction) from Arabidopsis thaliana registered in the Protein Data Bank with Protein Data Bank code “1gp6” (22) was used as a template of the initial structure for our investigation. A model with an ANS-ferryl (Fe(IV)/O/Fe(III)–O/H2O)-succinate-LCD complex for MD simulation was constructed as follows. The oxygen atom of the water molecule attached to the iron atom at the proximal position was substituted for the oxygen atom of the ferryl. Next, two LCDs were placed at the positions where the oxygenated product (quercetin 6) and the second substrate (enantiomer of DHQ 5) were located in the original crystal structure. The position occupied by a MES molecule was substituted with an ascorbate using the modeling software INSIGHT II (Accelrys, Inc., San Diego, CA). Finally, a rectangular water box (TIP3P model (23)) was generated with a width of 7 Å from ANS by the LEaP module of the AMBER 7.0 program package (24, 25). As a result, the ANS protein was solvated with 350 crystal water molecules and 7,507 additional water molecules.

The MD simulation was carried out using the AMBER 7.0 program package together with the Amber parm99 force field (26) except for the force field parameters associated with iron atom. Partial charges for the four residues coordinating to the iron atom of ferryl (Fe(IV)–O/Fe(III)–O) were calculated using the quantum chemical calculation by GAUSSIAN 98 (Gaussian, Inc., Carnegie, PA) (27) followed by reduced electrostatic potential fitting (28–30). The system was minimized for 4,000 steps prior to the MD simulation. The MD simulation was performed starting from the energy-minimized structure (shown in Fig. 2A). The temperature was elevated from 0 to 310 K during the first 60 ps of simulation, and then the temperature and pressure were kept at 310 K and 1 atm, respectively. The integration time step of the simulation was chosen to be 1 fs, and a cut-off distance of 10 Å for non-bonded interactions and a dielectric constant of ε = 1 were employed. A periodic boundary condition was applied, and the particle mesh Ewald method (31) was used for the estimation of long range electrostatic interactions. The system was equilibrated for 100 ps, and the average structure of the last 5 ps of the trajectory was saved as the protein structure in the physical condition (Fig. 2B). This average structure was used as a template for the following ab initio quantum chemical calculations.

**Analyses of Reaction Mechanisms by Quantum Chemical Calculations**—By theoretical approaches using quantum chemical calculation, we can obtain a variety of information such as structures of intermediates, potential energy surfaces, and a distribution of electron density. For example, we can reveal the activation energy and the direction of the reaction by...
FIGURE 3. Structures and potential surface at the hydrogen abstraction step. A, structure a, initial structure; structure b, structure after H3 abstraction; structure b', structure after H4 abstraction. B, potential surface at this step.
estimating the potential energy surface. Especially, it is difficult to experimentally reveal the unstable transition state structures or the intermediates in a rapid reaction. Quantum chemical calculations can predict these structures and provide additional information about the reaction. This is a fine advantage of this theoretical approach. In this study, the reaction mechanisms of the oxygenation of LCD 2 by ANS have been investigated in detail using this approach. We used the complex structure of LCD 2 and ANS obtained by the MD simulation as a starting structure. Obviously, the oxygenation of LCD 2, which is expected to be initiated with the abstraction of a hydrogen from C-3 or C-4, requires a multistep reaction. Thus, we have analyzed oxygenation at each site separately in a stepwise manner. In each reaction, we calculated the optimized structures both at the initial and the final states and further estimated the potential energy surface along each reaction. We used the density functional theory (DFT) as a method for quantum chemical calculations. DFT is an extremely successful approach for the description of ground state properties of metals, semiconductors, and insulators. The success of DFT not only encompasses standard bulk materials but also complex materials such as proteins and carbon nanotubes, recently. DFT methodology has been proved to be adequate for the calculation of a large variety of molecular properties (32, 33) as well as cost effective (34), and it has been widely used for studying enzymatic catalysis (35) including oxygenation (36, 37). It is difficult to treat all atoms of the large protein with the \textit{ab initio} quantum chemical calculation due to limitations of computational resources and time. For this reason, we constructed a truncated cluster model by extracting LCD 2, Fe(IV)=O, His\textsuperscript{232}, Asp\textsuperscript{234}, His\textsuperscript{288}, and succinate \textit{10}, which are considered to participate directly in the oxygenation process, from the average structure obtained by MD simulation. To construct the computational models, we replaced the backbone atoms of His\textsuperscript{232}, Asp\textsuperscript{234}, and His\textsuperscript{288} with hydrogen atoms, and the C–H bond distances were adjusted to a typical C–H bond length, 1.11 Å. Hence, in the present model, the Asp residue was replaced by CH\textsubscript{3}-COO\textsuperscript{−}, and His residues were replaced by C\textsubscript{6}H\textsubscript{4}N\textsubscript{2}−CH\textsubscript{3}. The total number of atoms was 81. The constructed models are shown in Figs. 3–6. Most of the three-dimensional structures of intermediates in ANS oxygenation process have not been yet determined by crystallographic or NMR experiments. Because the accuracy of reaction paths, potential surfaces, and structures obtained by quantum chemical calculations highly depends on the model system and the calculation method, it is very important to check the validity of the computations from the viewpoint of consistency with available experimental findings.

\textbf{Calculation Method}—We performed \textit{ab initio} quantum chemical calculations by using the B3LYP DFT to calculate the geometric and electrostatic structures of the ANS active site during the dioxygenation process. The DFT B3LYP functional is a hybrid functional constructed by combining Becke’s three-parameter exchange functional (38) and Lee-Yang-Parr’s correlation functional (39). Geometry optimizations were executed with a 3–21G\textsuperscript{−} basis set. During geometry optimization, atoms were allowed to move freely except for the CH\textsubscript{3} groups of three amino acid residues and the C-ring of the substrate, which are labeled with asterisks in Fig. 3A. These atoms were fixed at the position in the structure obtained by MD simulation during the optimization on each stationary point structure. Thus, the rigidity of the protein backbone and the sustainability of the substrate were taken into account in the model. Potential energy profiles were obtained by changing the distances shown on the horizontal axes in Figs. 3–6 in a stepwise manner. For example, the potential energy profile for the oxygenation initiating reaction, which is the abstraction of the hydrogen atom bound to the C-3 carbon atom by the ferryl oxygen (Fig. 3), was obtained by the following procedure. First, all structures of the reactant (Fig. 3A) and the product (Fig. 3B) were optimized. The distance between the abstracted hydrogen atom and the ferryl oxygen atom was gradually shortened, and the potential energies at the respective distance were calculated by the geometry optimization fixing the distance between the hydrogen atom and the ferryl oxygen atom. We repeated this process until the structure reached the product. Then, we plotted the energy changes as a function of distance. This energy profile will represent the potential energy surface along the hydrogen abstraction reaction. Using this procedure, we obtained a total of seven potential energy profiles for the oxygenation processes at the C-3 and C-4 positions of the substrate, which are shown in Figs. 3–6 and summarized in Fig. 7. Our calculations showed that the potential energy of the quintet state structure was lower than that of the triplet state structure. Hence, the calculations in the present study were performed in the quintet state. The total atomic charge was −1e.

\textbf{RESULTS}

\textit{Dynamic Structures of the Ferryl Species}—Molecular mechanics simulation afforded the initial structure for the molecular dynamics simulation of ANS with the natural substrate 2R,3S,4S-LCD 2, iron and dioxygen, as shown in Fig. 2A. There are two LCDs binding in the active site, although the second one may not be directly involved in any reaction. The first substrate is linked to Glu\textsuperscript{306} by a hydrogen bond. The carboxyl oxygen atoms of succinate \textit{14} lie in the anion hole formed by the side chains of four amino acid residues, Asn\textsuperscript{215}, Tyr\textsuperscript{217}, Arg\textsuperscript{298} and Ser\textsuperscript{300}. The distance between the C-4 carbon (C4) atom of the first LCD 2 and Fe (3.81 Å) was shorter than that between the C-3 carbon (C3) and iron (4.61 Å). The structure of an active site obtained by 100-ps MD simulation at 310 K is shown in Fig. 2B. The distances between iron and C4 or C3 had changed to 3.09 and 3.32 Å, respectively.

### Table 1

| Group of atoms | Structure | a | b | b' |
|---------------|-----------|---|---|---|
| Iron          |           | 4.1| 4.2| 4.3 |
| O1            |           | 0.25| 0.09| 0.07 |
| H3 (H4)       |           | (0.01)| (0.00)| (0.00) |
| Substrate     |           | 0.02| −0.99| −0.99 |
| Succinate     |           | −0.48| 0.45| 0.41 |
| Rest          |           | 0.15| 0.20| 0.22 |

\textbf{Oxidation Mechanisms by Anthocyanidin Synthase}
FIGURE 4. Structures and potential surface at the ketone formation step. A, structure c, structure after 3-ketone 11 formation; structure c’, structure after 4-ketone 5 formation. B, potential surface at this step.

Distance between O1 (hydroxyl oxygen) and hydroxyl hydrogen attached to C3 or C4 (angstrom)

Oxidation Mechanisms by Anthocyanidin Synthase
Initiation of Oxidation by ANS—The model structure for the quantum chemical calculation was constructed from the structure shown in Fig. 2B by the procedure described under “Materials and Methods.” The oxygen atom of ferryl species (Fe(IV) = O/Fe(III)−O) (O1), a center of oxygenation, interacted with the hydrogen atom at the C-3 position of LCD 2 (H3) with a distance of 3.93 Å and with the hydrogen atom at the C-4 position (H4) with a distance of 3.05 Å (Fig. 3A, structure a). Our results suggested that the O1 is ready for oxygenation at both the C-3 and C-4 positions of LCD 2, although the C-4 reaction is apparently more probable based on the observed distances. The spin density distribution of the optimized structure is shown in Table 1. O1 atom was found to have the character of a free radical. Since it is well known that a free radical abstracts a hydrogen atom from hydrocarbons (40, 46), it is plausible that the mechanism of oxygenation by ferryl species is initiated by hydrogen atom abstraction from the substrate interacting with the O1. As the distance between O1 and H3 or H4 atoms decreased, the potential energy of the reaction model increased (Fig. 3B), and a transition state structure appeared at the distance of 1.2 Å. Passing the transition state, the fifth ligand (O1) became an OH group, and the spin density was localized at the substrate radical in both H3 and H4 abstraction reactions (Fig. 3A, structures b and b′ and Table 1). Although the distance of the O1 atom was closer to the C-4 position than to C-3, the activation energies for the H4 and H3 abstraction reactions were similar, at ~31 and 32 kcal/mol, respectively (Fig. 3B). The energy of the C-3 radical structure (Fig. 3A, structure b) was ~11 kcal/mol higher than that of the C-4 radical structure (Fig. 3A, structure b′), indicating that proton abstraction could occur advantageously at the C-4 position of LCD 2.

Production of the 3-Ketone and Succinate Conformational Change in the C-3 Reaction—The C3 atom of the substrate forms a covalent bond with the oxygen atom (O1) at the proximal position to produce an oxygenated product from the structure obtained by hydrogen atom abstraction. Due to the approach of the hydroxyl oxygen (O1) to the C3 atom, the distance between O1 and the hydroxyl hydrogen attached to the C-3 position of LCD 2 became close, resulting in the production of water and 3-ketone 11 (2R,4S-flav-3-on-4-ol). The activation energy for this reaction is only ~1 kcal/mol (Fig. 4B). Sensitivity of the production of 3-ketone 11 (and water) occurs easily. The spin density distribution at the point of 3-ketone 11 formation (Table 2, column c) shows that the spin localized at the substrate in the C-3 radical (Table 2, column b) diminished by the production of the water molecule. This structure seems to be stable; however, the C3 atom of LCD 2 must bind to the O1 atom to produce an oxygenated substrate, which may be achieved by the nucleophilic addition of hydroxyl oxygen (O1). The H3 atom of water molecule was abstracted by the oxygen atom of the succinate 14, implying that succinate 14 functions as a Lewis acid, and the nucleophilic addition of hydroxyl anion to the C-3 position follows. The potential energy transitions that occur along with the alteration of the distance between O1 and C3 are shown in Fig. 5B. The present calculations revealed that this oxygen addition could not occur in a single-step process but required double steps. First, the conformation of succinate 14 changes with the decrease in the distance between O1 and C3 (Fig. 5A, structure d). The OH bound intermediate is then produced by the further decrease in this distance (Fig. 5A, structure e). This result indicates that the conformation change of succinate 14 increases its own pKₐ value, which is essential in order for it to function as a Lewis acid. Table 2 shows that the spin density distribution did not change in these reactions (c → d → e), and that the d → e reaction was caused by the nucleophilic addition. The activation energies for the d → e and e → f reactions were 6 and 11 kcal/mol, respectively.

Production of 3,3-Geminal Diol—Since the oxygen atom attached to C3 (O3) (Fig. 5A, structure e) had the character of an anion, it is anticipated that the more stable structure is produced by the addition of H⁺. The potential energy transition with a decrease in the distance between O3 and H3 is shown in Fig. 6B. We found that 3,3-gem-diol 10 (2R,45-flavan-3,3,4-triol) was formed by the addition of H⁺ (H3) to O3 (Fig. 6A, structure f), with an activation energy of ~3 kcal/mol.

Reaction at the C-4 Position—With respect to the reaction at the C-4 position, 4-ketone 5 (2R,3R-trans-DHQ) was formed by the approach of O1 and the hydroxyl hydrogen attached to C4 (Fig. 4A, structure c′). Spin transfer from the substrate also occurred in this reaction (Table 2), with an activation energy of ~1 kcal/mol (Fig. 4B). The potential energy of structure c′ was ~18 kcal/mol more stable than that of structure c. Since the H4 atom was attracted by the oxygen atom of succinate 14 in the C-4 reaction, nucleophilic addition by the O1 hydroxyl oxygen atom is also anticipated. However, neither the conformational change of succinate 14 nor OH⁻ addition occurred with the approach of C4 and O1 (Fig. 5B).

DISCUSSION

The potential energy change through the reaction is summarized in Fig. 7. The potential energy of the 3,3-gem-diol 10 structure (f) was estimated to be ~16 kcal/mol lower than that of the starting structure (a). The structure containing the 4-ketone (2R,3R-trans-DHQ 5) (c′) has also lower potential energy than the starting structure (a). Furthermore, both of them have the lowest energies in their reaction paths: oxygenation processes at both C-3 and C-4 positions. These indicate that the productions of the 4-ketone 5 and the 3,3-gem-diol 10 are energetically favorable, and the reactions proceed in the direction of production. The activation energies for hydrogen atom abstraction reactions were estimated to be ~32 and 31 kcal/mol in the reactions at C-3 and C-4 positions, respectively. These abstraction reactions were proved to be the rate-limiting steps in their oxygenation processes. These values are too high and seem to be overestimated. Despite this, the findings that the rate-limiting step in the LCD 2 oxygenation

TABLE 2
Spin density distributions for each structure obtained in the oxygenation steps

| Group of atoms | Structure | b | c | d | e | f | b′ | c′ |
|---------------|-----------|---|---|---|---|---|----|----|
| Iron          |           | 4.24 | 3.86 | 3.87 | 3.87 | 3.86 | 4.29 | 3.90 |
| O1            |           | 0.99 | 0.00 | 0.00 | 0.00 | 0.00 | 0.07 | 0.04 |
| Substrate     |           | −0.99 | 0.28 | 0.00 | 0.00 | 0.00 | −0.99 | −0.35 |
| Succinate     |           | 0.45 | −0.23 | 0.06 | 0.05 | 0.07 | 0.41 | 0.30 |
| Rest          |           | 0.20 | 0.08 | 0.06 | 0.07 | 0.05 | 0.22 | 0.10 |

See Figs. 3A, 4A, 5A, and 6A for details on the structures below.
FIGURE 5. Structures and potential surface at the hydroxylation step. A, structure d, structure after the conformation change of succinate 14; structure e, structure after OH addition. B, potential surface at this step.
process is the hydrogen atom abstraction reaction is consistent with several previous theoretical studies on the oxygenation mechanism by the ferryl \((\text{Fe(IV)}=\text{O}/\text{Fe(III)}–\text{O})\) species of cytochrome P-450 (36, 41, 42). Hence, the potential energy surfaces obtained in this study is considered to reflect the actual physiological phenomenon and the oxygenations at both C-3 and C-4 positions by ANS occur spontaneously under the condition of room temperature. It is well investigated experimentally (40, 46) and theoretically (36, 41, 42) that ferryl species have high reactivity. Thus, the excess enthalpy for the oxygenation process may be supplied by the production of ferryl species, which precedes the oxygenation of substrate.

In the oxygenation reaction at the C-3 position of \(2R,3S,4S\)-cis-LCD (structure a), H3 atom is initially abstracted by O1 to form C-3 radical intermediate (structure b) followed by 3-ketone 11 and water molecule formation via additional hydrogen abstraction from the hydroxyl group at the C-3 position (structure c). It was possible to change the conformation of the succinate molecule 14 associated with Fe so that it would accept \(\text{H}^+\) from water (structure d), and the generated hydroxyl anion could then be attached at the C-3 position (structure e). Although the succinate 14 generally formed in the first step of the 2-oxoglutarate-dependent dioxygenase reaction has been regarded as a mere side product, the present results suggest that succinate molecule 14 can play an important role in the oxygenation reaction. The proton retained on the succinate 14 then returns to carbonyl oxygen (O3), that is, it forms 3,3-gem-diol 10 (structure f). On the other hand, C-4 oxidation is also initiated with C-4 radical formation by H4 atom abstraction (\(b'\)) followed by the formation of 4-ketone 5 and water molecule (\(c'\)). However, no other reactions can occur, since the 4-ketone (\(2R,3R\)-trans-DHQ 5) is a stable compound. Our calculations demonstrated that it is possible for both C-3 and C-4 oxidations to occur, but that the C-4 reaction has an energetic advantage, suggesting that C-4 oxidation by ANS can occur preferentially \textit{in vivo}, and the C-3 reaction, which synthesizes anthocyanin pigments (e.g. 1), might be simply a side reaction. The present results are consistent with a recent \textit{in vitro} study which showed that the major product (85%) of oxygenation on natural \(2R,3S,4S\)-cis-LCD 2 by \textit{Arabidopsis} ANS was not cyanidin 1 but quercetin 6 (11).

Fig. 8 shows the anticipated \textit{in vivo} reactions on \(2R,3S,4S\)-cis-LCD 2 by ANS. \(2R,3R\)-trans-DHQ 5 formation by direct C-4
Oxidation takes place preferentially (Fig. 8, I'). The C-3 position of DHQ 5 can be reoxygenated by ANS to form quercetin 6 via an exchange of oxygen probably brought about by equilibration between 3-ketone and 3,3-gem-diol (II') (17). The present result that 4,4-gem-diol could not form in the C-4 reaction is consistent with our previous in vitro biochemical experiment, in which it was found that there was no incorporation of oxygen atom from solvent at the C-4 position (17). On the other hand, C-3 oxygenation of 2R,3S,4S-cis-LCD 2 to form 3,3-gem-diol 10 via equilibration with 3-ketone 11 can also occur, although it may be a side reaction (Fig. 8, I). Since the hydrogen conformation at the C-2 and C-4 positions of 3,3-gem-diol 10 derived from 2R,3S,4S-cis-LCD 2 are axial and equatorial, respectively, dehydration from the C-2 and C-3 positions preferentially occurs to form 4S-flav-2-en-3,4-diol 4 (Fig. 8, II) (11). The C-3 position of the 3,4-diol 4 is expected to be glycosylated in vivo by FGT, after which it is transported into acidic vacuoles to form colored flavylum ion of cyanidin 3-glycoside (e.g. 13) (Fig. 8, III) (9). However, if 4S-flav-2-en-3,4-diol 4 is still held at the active site of ANS, quercetin 6 might be formed by the reoxygenation of either the C-3 or C-4 position (Fig. 8, IV) (11, 17). The metabolic stream in the biosynthetic pathway of anthocyanin might flow toward quercetin 6 as described above. Recently, anthocyanidin reductase was found to catch anthocyanidins (e.g. 1) as their substrate to form 2R,3R-cis-flavan-3-ol (e.g. epicatechin 15) (Fig. 8, V) (43). Since the catalysis of anthocyanidin reductase may occur in cytoplasm (that is, not...
under acidic conditions) as other biosynthetic enzymes, flav-2-en-3,4-diol 4, the pseudobase form of anthocyanidins (e.g. 1), is likely to participate in epicatechin synthesis.

Unnatural 2R,3S,4R-trans-LCD can also serve as a substrate for Arabidopsis ANS although different product distribution was observed in the case of natural LCD 2, i.e. major product (55%) was cis-DHQ 7 (11). Our calculation, that most of the first oxidation reaction takes place on C-4 of cis-LCD 2 (initiated by abstraction of H4) to form trans-DHQ 5 directly, also supports the experimental result. Namely, not C-4 but C-3 oxygenation might be preferable in the reaction on trans-LCD since H4 atom of trans-LCD might be harder to access than that of cis-LCD 2.

In nature, 2R,3S,4R-trans-dihydroflavonols (e.g. 5) can be reduced by dihydroflavonol reductase (DFR) and NADPH to 2R,3S,4S-cis-LCDs (e.g. 2), followed by ANS oxidation. The present study and other recent in vitro studies suggest that most cis-LCDs (e.g. 2) might return to trans-dihydroflavonols (e.g. 5) by C-4 reoxidation by ANS even though some of the product can follow the biosynthetic pathway, bringing about pigment formation (10–12, 17, 44). This mechanism appears to be a case of wasteful spending of NADPH, 2-oxoglutarate 3 and oxygen, but actually, trans-dihydroflavonol (e.g. 5, the substrate for DFR) is an isomer of flav-2-en-3,4-diol 4 (a direct product of ANS). If continuous tautomerization from trans-dihydroflavonols (e.g. 5) to 4S-flav-2-en-3,4-diol 4 via flav-3-en-3,4-diol 12 and flav-3-on-4-ol 11 is possible, the steps catalyzed by DFR and ANS can be bypassed (Fig. 9). However, at least the tautomerization step from flav-3-en-3,4-diol 12 to 4S-flav-2-en-3,4-diol 4 is energetically impossible (45), with the result that the bypass cannot exist. Higher plants use two enzymes (DFR and ANS), NADPH, 2-oxoglutarate 3, and dioxygen to overcome such energetically disadvantageous steps.

FIGURE 9. Possible bypass from dihydroflavonol to anthocyanidin.

ANS may have evolved from FLS although the evolution is not yet complete. FLS does not have stereoselectivity for its oxygenation since the natural substrate for FLS, 2R,3R-trans-dihydroflavonol (e.g. 5), is a 4-ketone compound; that is, the C-4 position cannot be further oxidized. Before plants started to produce anthocyanins (e.g. 13), 2R,3S,4S-cis-LCDs (e.g. 2), the product of DFR, may have existed as a precursor for catechin biosynthesis. The cis-leucoanthocyanidins (e.g. 2) might have been oxidized by FLS accidentally, resulting in the production of C-2 and C-3 desaturated compound 4S-flav-2-en-3,4-diols 4. The 3,4-diols 4 might have been glycosylated at the C-3 position by FGT to form a stable intermediate. Then the glycosylated diols might have been changed chemically to colored flavylum cation form of anthocyanins (e.g. 13) in an acidic environment when transported into vacuoles like other flavonoids. Higher plants with acquired pigments may prosper due to the greater attraction for their pollinators.

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