Reaction Mechanism from Leucoanthocyanidin to Anthocyanidin 3-Glucoside, a Key Reaction for Coloring in Anthocyanin Biosynthesis*

Received for publication, January 26, 2001, and in revised form, April 20, 2001

Published, JBC Papers in Press, April 20, 2001, DOI 10.1074/jbc.M100744200

Jun-ichiro Nakajima‡, Yoshikazu Tanaka§, Mami Yamazaki‡, and Kazuki Saito‡¶

From the ‡Department of Molecular Biology and Biotechnology, Graduate School of Pharmaceutical Sciences, Chiba University, Yayoi-cho 1-33, Inage-ku, Chiba 263-8522, Japan and the §Institute for Fundamental Research, Suntory Ltd., Wakayama-dai 1-1-1, Shimamoto, Mishima, Osaka 618-8503, Japan

In the conversion from colorless leucoanthocyanidin to colored anthocyanidin 3-glucoside, at least two enzymes, anthocyanidin synthase (ANS) and UDP-glucose: flavonoid 3-O-glucosyltransferase (3-GT), are postulated to be involved. Despite the importance of this reaction sequence for coloring in anthocyanin biosynthesis, the biochemical reaction mechanism has not been clarified, and the possible involvement of a dehydratase has not been excluded. Here we show that recombinant ANSs from several model plant species, snapdragon, petunia, torenia, and maize, catalyze the formation of anthocyanidin in vitro through a 2-oxoglutarate-dependent oxidation of leucoanthocyanidin. Crude extracts of Escherichia coli, expressing recombinant ANSs from these plant species, and purified recombinant enzymes of petunia and maize catalyzed the formation of anthocyanidin in the presence of ferrous ion, 2-oxoglutarate, and ascorbate. The in vitro formation of colored cyanidin 3-glucoside from leucocyanidin, via a cyanidin intermediate, was demonstrated using petunia ANS and 3-GT. The entire reaction sequence did not require any additional dehydratase but was dependent on moderate acidic pH conditions following the enzymatic steps. The present study indicated that the in vivo cytosolic reaction sequence involves an ANS-catalyzed 2-oxoglutarate-dependent conversion of leucoanthocyanidin (flavan-3,4-cis-diol) to 3-flavan-2,3-diol (pseudobase), most probably through 2,3-desaturation and isomerization, followed by glucosylation at the C-3 position by 3-GT.

The reaction leading from colorless leucoanthocyanidin to anthocyanidin and its 3-O-glucoside is the critical step in the formation of colored metabolites in anthocyanin biosynthesis (1–3) (Fig. 1). Although anthocyanidin is the first colored metabolite in the biosynthetic pathway, it is hardly detected in plant tissues because of its instability. Anthocyanidin 3-glucoside is the first stable colored metabolite detected in plants. The entire reaction formally involves dehydrogenation at C-2, dehydration at C-3 and C-4, and glucosylation at the hydroxyl group of C-3.

Anthocyanidin synthase (ANS)1 is presumed to catalyze the first half of the reaction involving dehydrogenation at C-2 and dehydration at C-3 and C-4. cDNAs and genes that presumably encode ANS have been isolated from several plant species (4–12). The involvement of ANS in this step is supported by the ability of a cDNA encoding maize ANS to restore anthocyanin formation in an ANS- mutant of maize, through transient expression by particle bombardment (4). Sequence comparison with deduced amino acid sequences of these ANSs suggested that they belong to a family of soluble oxygenases depending upon 2-oxoglutarate (13, 14).

The precise biochemical reaction mechanism from leucoanthocyanidin to anthocyanidin has not been clarified to date. As shown in Fig. 2, three possible mechanisms have been postulated for the reaction sequence from leucoanthocyanidin to 3-flavan-2,3-diol (pseudobase), which is chemically converted into anthocyanidin (flavylum ion) by removal of the C-2 hydroxyl anion. In the first mechanism (2) (Fig. 2, pathway A), ANS catalyzes a desaturation at the C-2 and C-3 positions of (2R,3S,4S)-leucoanthocyanidin (15, 16) in the presence of 2-oxoglutarate and molecular oxygen, yielding 2-flavan-3,4-diol, with concomitant release of CO₂, succinate, and H₂O. After isomerization of the 2,3-double bond to the 3,4-position of 2-flavan-3,4-diol, by removal of the hydroxy group at C-4, double bond migration, and insertion of a hydroxyl group at C-2, 3-flavan-2,3-diol is formed. Alternatively, ANS may catalyze a hydroxylation at the C-2 position of leucoanthocyanidin, followed by 3,4-dehydration (1) (Fig. 2, pathway B)). In the third postulation, 3,4-dehydration occurs as the first step, followed by a hydroxylation at C-2 (1) (Fig. 2, pathway C)). The last two assumptions (pathways B and C) imply that the formation of 3-flavan-2,3-diol may require not only ANS but also a specific dehydratase catalyzing 3,4-dehydration. Alternatively, ANS may catalyze the dehydration of the reaction. So far there is no evidence to suggest the involvement of an external dehydratase in the whole reaction sequence, except for a preliminary report describing a genetic locus that possibly encodes a dehydratase in snapdragon (Antirrhinum majus) (17).

To date, in vitro biochemical analysis regarding this crucial reaction for anthocyanin biosynthesis has only been carried out with recombinant ANS from Perilla frutescens (10). Recombinant perilla ANS was shown to catalyze the formation of anthocyanidin from leucoanthocyanidin according to a 2-oxoglutarate-dependent manner when followed by an acidification

* This work was supported in part by grants-in-aid for scientific research from the Ministry of Education, Science, Sports, and Culture, Japan, by a grant from the Research for the Future Program (96FI00932) from the Japan Society for the Promotion of Science, Japan, by CREST of Japan Science and Technology, by the Ho-an-sha Foundation, Osaka, Japan, and by San-Ei Gen Foundation, Osaka, Japan. 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.

† To whom correspondence should be addressed. Tel./Fax: 81-43-290-2905; E-mail: ksaito@p.chiba-u.ac.jp.

1 The abbreviations used are: ANS, anthocyanidin synthase; 3-GT, UDP-glucose:flavonoid 3-O-glucosyltransferase; PCR, polymerase chain reaction; MBP, maltose-binding protein; SDS-PAGE, SDS-polyacrylamide gel electrophoresis; HPLC, high performance liquid chromatography.
Fig. 1. Formal reaction from colorless leucoanthocyanidin to colored anthocyanidin 3-glucoside. The reaction formally involves dehydrogenation at C-2, dehydration at C-3 and C-4, and glucosylation at the C-3 hydroxyl group.

with HCl. It is necessary to verify this important reaction mechanism with ANSs from various model plant species for anthocyanin biosynthesis under in vivo conditions, where ANS is combined with a UDP-glucose:flavonoid 3-O-glucosyltransferase (3-GT) at cytosolic and vacuolar pH.

The second half of the reaction (Fig. 1), the formation of anthocyanidin 3-glucoside from anthocyanidin, has also not been completely clarified. From intertissue complementation assays in maize (18) it has been generally assumed that leucoanthocyanidin first is converted to 3-flaven-2,3-diol (pseudobase) by ANS and is then glucosylated by 3-GT (1). No in vitro biochemical experimental evidence has been provided to confirm the reaction sequence from leucoanthocyanidin to anthocyanidin 3-glucoside, although 3-GT activity has been detected in crude enzyme preparations of various plant species (19–22) and in preparations containing recombinant 3-GT (23, 24). The reaction sequence catalyzed by ANS and 3-GT is postulated to occur in the cytosol, followed by transport into vacuoles, where the colored anthocyanin (flavonoid ion) is formed as a consequence of the acidic pH (25). However, no in vitro experimental evidence for this hypothesis has been provided yet. Therefore, it is also necessary to examine whether anthocyanidin 3-glucoside can be formed from leucoanthocyanidin using both ANS and 3-GT recombinant proteins under physiological conditions, where enzyme reactions take place under cytosolic conditions (pH 7) followed by a shift to vacuolar conditions (pH 5) (26).

In the present study, we expressed and functionally verified, for the first time, four recombinant ANSs from snapdragon (A. majus), petunia (Petunia hybrida), torenia (Torenia fournieri), and maize (Zea mays). The reaction catalyzed by these ANSs was shown to proceed via a 2-oxoglutarate-dependent oxygenation of leucoanthocyanidin in the formation of anthocyanidin. Evidence, which implicates that ANS catalyzes the reaction from leucoanthocyanidin to anthocyanidin in the absence of an external dehydratase or strong acid conditions, is provided. We show that the combination of recombinant ANS and 3-GT was sufficient and essential to yield cyanidin 3-glucoside from leucoanthocyanidin under physiological conditions, by mimicking cytosolic enzyme reactions and transport into vacuoles.

EXPERIMENTAL PROCEDURES

cDNA Clones from Various Model Plant Species—The cDNA clones Candi and A2, encoding ANS of A. majus (5) and Z. mays (4), were kind gifts from Dr. Cathie Martin and Dr. Alfons Gierl, respectively. The cDNA clone TAN1, encoding ANS from T. fournieri cv. Summer Wave Blue (Suntory Ltd.) was isolated as described previously (12). The cDNA encoding petunia ANS was isolated by PCR screening of a cDNA library constructed from P. hybrida cv. Surfina (Suntory Ltd.) using the forward primer 5'-CCG GAA TTC ATG GTG AAT GCA GTA GTT-3' and the reverse primer 5'-GCT CTA GAA CTC GTG ATT CCA ACA CAT CAT C-3'. Amplification was carried out for 25 cycles at the following conditions: 94 °C/0.5 min, 55 °C/1.5 min, 72 °C/2.8 min. The nucleotide sequence was verified by sequence analysis using the dideoxy chain termination method with Thermo Sequenase (Amersham Pharmacia Biotech) using a DNA sequence (model DSQ-2000L; Shimadzu, Kyoto, Japan) and was shown to be identical with the published sequence (6). The cDNA clone PGT8, encoding petunia 3-GT (GenBankTM accession number AB027454), was isolated from P. hybrida cv. Surfina.2

2 Yamazaki et al., submitted for publication.
**Mechanism of Anthocyanin Formation**

**XbaI site** was created at the 5' and 3'-terminal ends, respectively, of the coding region of ANS by PCR amplification with each primer set as follows: for snapdragon ANS, 5'-CCG GAA TTC ATG GCA CCG GCA ATA GTC CCA CTC CCC TGG G-3' and 5'-GCT GTA GAT TCA ACA TTT CTT TCT TGC TCT GTA GCA-3' for torenia ANS; 5'-CCG GAA TTC ATG GTC TCT CCA GCA TCT CCG ACC GCG C-3' and 5'-GCT CTA GAC TCA ACA CTA TTA TCA TGC TCA ACA-3'; for maize ANS, 5'-CCG GAA TTC ATG GAC TCG TCG CTC CGT CTG-3' and 5'-GCT CTA GAT CAG TGT GTC TGC GGC GGC GCC-3'. For petunia ANS, an EcoRI site and XbaI site were created by PCR screening using the primer set as above. The amplified fragments were ligated into the EcoRI-XbaI site of pMAL-c2 (New England Biolabs, Beverly, MA) to afford pMDSA1 (snapdragon), pMPTA9 (petunia), pMTOA6 (torenia), and pMMZA3 (maize), respectively. An XbaI site and PstI site were created at the 5’- and 3’-terminal ends of the coding region of petunia 3-GT by PCR amplification with the primer set as above. The amplified fragment was ligated into the XbaI-PstI site of pMAL-c2 to afford p0GTM7. The ANSs and 3-GT were expressed as fusion proteins with maltose-binding protein (MBP, 42.7 kDa) at the N terminus. Each vector was introduced into *E. coli* Nova Blue for overexpression.

**Production and Purification of Recombinant Enzymes—*E. coli* Nova Blue** carrying recombinant ANS (pMDSA1, pMPTA9, pMTOA6, and pMMZA3) and recombinant 3-GT (pGTM7) was cultured in the liquid medium (20 mM potassium phosphate buffer, pH 7.0, 200 mM NaCl, 5 mM dithiothreitol, 15 g/liter) supplemented with glucose (2 g/liter) and ampicillin (100 mg/liter) at 37 °C. Gene expression was induced by addition of 0.3 mM isopropyl-β-D-thiogalactopyranoside at 28 °C for 3 h. The cells were centrifuged at 10,000 × g for 10 min, and the supernatants were stored at −20 °C. Cell pellets were disrupted by sonication in Buffer B (20 mM potassium phosphate buffer, pH 7.0, 200 mM NaCl, 5 mM dithiothreitol, 10% glycerol) according to the manufacturer's protocol. All purification procedures were performed at 4 °C.

**SDS-Polyacrylamide Gel Electrophoresis (PAGE) and Western Blotting—**SDS-PAGE was carried out using 8% polyacrylamide gels as described previously (27). Western blotting and immunostaining were performed on an Immobilon P membrane (Millipore) using phosphatase-labeled goat anti-rabbit IgG (Kirkegaard & Perry Laboratories) and horseradish peroxidase-conjugated goat anti-rabbit IgG. The membranes were visualized with Eastern Blue carrying recombinant ANS from petunia, and maize and were then subjected to amyllose resin column chromatography (New England Biolabs) in Buffer B (20 mM potassium phosphate buffer, pH 7.0, 200 mM NaCl, 10 mM maltose, 5 mM dithiothreitol, 10% glycerol) according to the manufacturer's protocol. All purification procedures were performed at 4 °C.

**RESULTS**

**Heterologous Expression of ANS and 3-GT in *E. coli*—**The open reading frames of cDNAs encoding ANS from snapdragon, torenia, and maize and the cDNA encoding 3-GT from petunia were amplified by PCR and subcloned into pMAL-c2 expression vectors under the control of the tac promoter. The constructs carrying ANS-encoding cDNA from snapdragon, torenia, and maize and 3-GT-encoding cDNA from petunia were named pMDSA1, pMTOA6, pMMZA3, and pGTM7, respectively. The construct, pMPTA9, was generated by subcloning the coding region of the petunia ANS-encoding cDNA, including the 78-base pair nucleotide sequence of the 3'-flanking region, into pMAL-c2. The vectors were used to transform *E. coli* Nova Blue. The empty vector, pMAL-c2, was applied as a negative control, and pMK1, carrying the ANS-encoding cDNA from perilla (10), was introduced into *E. coli* as a positive control for the expression of catalytically active recombinant protein. The ANSs and 3-GT were overexpressed in *E. coli* as fusion proteins with MBP and exhibited a greater molecular mass than expected due to the presence of MBP (42.7 kDa).

Soluble protein extracts of the transformed *E. coli* were separated by SDS-PAGE and stained with Coomassie Brilliant Blue.
Mechanism of Anthocyanin Formation

The reaction and HPLC analysis were carried out as described under "Experimental Procedures". A, standard cyanidin. B, reaction product from leucocyanidin. C, co-chromatography of the reaction product (B) with standard cyanidin. D, reaction product by the protein extract of control E. coli transformed with an empty vector (pMAL-c2). E, standard pelargonidin. F, reaction product from leucopelargonidin under the same condition as for (B).

Cyanidin was formed by extracts of E. coli expressing recombinant ANS from all of the different plant species (Fig. 5), although cyanidin formation did not show linearity with time after 10 min of incubation, most likely because synthesized cyanidin decomposed due to an inherent instability or because ANSs were inactivated during the incubation.

Purified recombinant MBP-ANSs also exhibited ANS activity and were able to catalyze the formation of cyanidin (Table I). The specific activities for the formation of cyanidin were 0.972 and 0.215 microkatal/kg of protein for petunia MBP-ANS and maize MBP-ANS, respectively, obtained by initial velocities of 5 min of incubation. These results indicated that, for the conversion of leucoanthocyanidin to anthocyanidin, only ANS is required, and no additional dehydratase is needed.

Enzyme Properties of ANS as a 2-Oxoglutarate-dependent Oxygenase —The cofactor requirements of the purified recombinant ANSs from petunia and maize were investigated, and the results are summarized in Table I. The formation of cyanidin was absolutely dependent on the presence of ferrous ion, ascorbate, and 2-oxoglutarate. When O₂ was substituted with N₂, cyanidin production was not observed. These results clearly indicate that ANS belongs to a family of 2-oxoglutarate-dependent oxygenases requiring O₂ and ferrous ion. In addition, no dehydrated compound was produced upon incubation of leucoanthocyanidin with purified petunia ANS in the absence of cofactors (data not shown), suggesting that ANS itself has no dehydration activity toward leucoanthocyanidin.

The Entire Reaction Sequence Catalyzed by ANS and 3-GT —To clarify the entire reaction sequence between leucoanthocyanidin and anthocyanidin 3-glucoside, we examined combinatory and sequential reactions involving both recombinant ANS and 3-GT in vitro. Fig. 6A shows the reaction products obtained from leucoanthocyanidin by the combined presence of ANS and 3-GT together with the appropriate cofactors for both enzymes (2-oxoglutaric acid, sodium ascorbate, FeSO₄, and UDP-glucose). Both cyanidin and cyanidin 3-glucoside were formed. Upon the sequential reaction of ANS followed by 3-GT, where the intermediates were only exposed to one enzyme at a
Mechanism of Anthocyanin Formation

Cofactor requirement of the reaction catalyzed by purified MBP-ANS

For determination of initial velocities, the reaction for cyanidin formation was carried out for 5 min at 30 °C. Complete reaction mixture (100 μl) contained 20 mM potassium Pi (pH 7.0), 200 mM NaCl, 5 mM dithiothreitol, 4 mM sodium ascorbate, 1 mM 2-oxoglutarate, 0.4 mM FeSO₄, 1 mM (2R,3S,4R)-leuco cyanidin, and 100–200 μg of purified MBP-ANS protein. Ascorbate, FeSO₄, 2-oxoglutarate, or O₂ (replaced with N₂) was omitted from the complete reaction mixture. Boiled enzyme, the purified MBP-ANS, was heat-treated at 95 °C for 10 min prior to assay. Data are mean values of triplicate incubations ± S.D.

| Condition          | Enzymatic activity (μkat/kg) |
|--------------------|-----------------------------|
|                    | petunia ANS | maize ANS |
| Complete           | 0.972 ± 0.02 | 0.215 ± 0.01 |
| − Ascorbate        | <0.001 | <0.001 |
| − Ferrous ion      | <0.001 | <0.001 |
| − 2-oxoglutarate   | <0.001 | <0.001 |
| − O₂               | <0.001 | <0.001 |
| Boiled enzyme      | <0.001 | <0.001 |

**Fig. 6.** HPLC analysis of reaction products from leuco cyanidin by the combined presence of ANS and 3-GT. The reaction and HPLC analysis were carried out as described under “Experimental Procedures.” A, reaction products from leuco cyanidin by the coexistence of recombinant petunia ANS and petunia 3-GT after 30 min of incubation. B, reaction products by sequential reaction of ANS as the first enzyme for 30 min, followed by removal of ANS, and reaction of 3-GT as the second enzyme for an additional 30 min. C, reaction product of the opposite sequential reaction of 3-GT as the first enzyme, followed by removal of 3-GT, and ANS as the second enzyme. D, standard cyanidin 3-glucoside. E, standard cyanidin.

**Fig. 7.** Ultraviolet and visible absorption spectra of reaction products from leuco cyanidin by the combined presence of ANS and 3-GT. A, standard cyanidin 3-glucoside in pH 2, 5, and 7. B, reaction product by combination of ANS and 3-GT from leuco cyanidin. The product composition was confirmed to be ~90% cyanidin 3-glucoside and ~10% cyanidin by HPLC analysis. After removal of ANS and 3-GT by ultrafiltration, the pH of the incubation mixture was changed from 7 to 2 or 5. The reaction and determination of absorption spectra were carried out as described under “Experimental Procedures.” ABS, absorbance.

**TABLE I**

Cofactor requirement of the reaction catalyzed by purified MBP-ANS

| Condition          | Enzymatic activity (μkat/kg) |
|--------------------|-----------------------------|
|                    | petunia ANS | maize ANS |
| Complete           | 0.972 ± 0.02 | 0.215 ± 0.01 |
| − Ascorbate        | <0.001 | <0.001 |
| − Ferrous ion      | <0.001 | <0.001 |
| − 2-oxoglutarate   | <0.001 | <0.001 |
| − O₂               | <0.001 | <0.001 |
| Boiled enzyme      | <0.001 | <0.001 |

**DISCUSSION**

Most reactions involved in anthocyanin biosynthesis have been biochemically characterized with purified enzyme preparations and recombinant proteins (31, 32). However, the reactions from leuco anthocyanidin to anthocyanidin 3-glucoside (Fig. 1) have not been well characterized. In particular, the reaction catalyzed by ANS has not been well documented, because the detection of ANS activity in vitro has not been possible with crude enzyme preparations or recombinant proteins. Our recent study on *P. frutescens* ANS was the only evidence provided for the formation of anthocyanidin by ANS (10), although this study could not completely exclude the possible involvement of an external dehydratase in a variety of model plant species. The present study indicated that all tested putative ANSs (not only perilla ANS) were capable of catalyzing the 2-oxoglutarate-dependent reaction that converts leuco anthocyanidin to an oxidized metabolite, in the absence of additional enzyme(s) such as a dehydratase, which can be readily converted to anthocyanidin by acidification. Furthermore, the inclusion of an in vitro 3-GT reaction and the effect of cosylation of anthocyanidin as the second step. More importantly, only two enzymes, ANS and 3-GT, are necessary and sufficient for the catalysis of the sequential reactions leading from leuco anthocyanidin to anthocyanidin 3-glucoside. Therefore, no additional enzyme(s) such as dehydratase are required to complete the entire reaction sequence.

**Mimicking the in Vivo Sequential Reactions in Vitro—The in vivo conditions involved in the transport of anthocyanidin 3-glucoside into vacuoles were mimicked in vitro, whereby the sequential reactions of ANS and 3-GT yielding cyanidin 3-glucoside as ~90% of the products were followed by a shift in pH. After the incubation with both enzymes at pH 7 (cytosolic pH), the enzymes were removed by ultrafiltration, and a part of the reaction mixture was acidified to pH 2 or 5 (vacuolar pH) or maintained at pH 7 (cytosolic pH). The ultraviolet and visible spectra of all of the reaction products under these pH conditions were identical with those of standard cyanidin 3-glucoside (Fig. 7). These results indicate that the initial reaction product of ANS and 3-GT under neutral cytosolic conditions most likely is the pseudobase of anthocyanidin 3-glucoside and that the flavilyium ion of anthocyanidin 3-glucoside is formed after the pseudobase has been transported into the acidic environment of vacuoles (pH 5).
a pH shift that mimics transport of the glucoside into vacuoles have provided evidence for a plausible in vivo reaction sequence (as shown in Fig. 8). Namely, the 3-GT reaction takes place after the ANS reaction, and the actual substrate of the 3-GT is therefore most likely the pseudobase form of anthocyanidin present at cytosolic pH (pH 7), not the leucoanthocyanidin. The pseudobase form of anthocyanidin 3-glucoside is then transported into vacuoles, where the colored flavylum ion of anthocyanidin 3-glucoside is formed as a result of the acidic pH conditions of vacuoles.

In the flavonoid biosynthetic pathway, there are four reactions that are catalyzed by 2-oxoglutarate-dependent oxygenases: ANS (10, present study), flavanone 3-hydroxylase (33, 34), flavonol synthase (35), and flavone synthase I (36). All these reactions are concerned with oxidation at the C-2 and/or C-3 position of the flavonoid skeleton. In the reaction of flavone synthase I, the direct double bond formation at C-2 and C-3 of flavanone was proposed (36). Because no stable hydroxylated intermediates were detected in the reactions of ANS, flavonol synthase, or flavone synthase I, the mechanism involving direct 2,3-desaturation seems to be the most likely route for the formation of 2-flaven-3,4-diol (Fig. 8). Regarding the reaction mechanism of flavone synthase I, a radical mechanism was proposed for the direct 2,3-dehydrogenation of flavanone (36). In the proposed pathway A (Fig. 2) for the formation of the key compound, 2-flaven-3,4-diol, three mechanisms are possible: (i) direct dehydrogenation from C-2 and C-3, probably by a radical mechanism; (ii) hydroxylation at C-2 of leucoanthocyanidin to give flavan-2,3,4-triol and subsequent 2,3-dehydration to 2-flaven-3,4-diol (a modified pathway B in Fig. 2); (iii) hydroxylation at C-3 to give flavan-3,3,4-triol, spontaneous dehydration at 3-oxide to 2-flaven-3,4-diol, and subsequent keto-enol isomerization to 2-flaven-3,4-diol. However, the route via flavan-2,3,4-triol (ii) may require a dehydratase, either leading to 2-flaven-3,4-diol or to 3-flaven-2,3-diol. If this dehydration indeed takes place, it is now evident that no external dehydratase is required, although we cannot completely exclude the possibility that ANS itself may catalyze the dehydration of flavan-2,3,4-triol (pathway B in Fig. 2, for example). Because the present study provided evidence that ANS does not catalyze the dehydration of leucoanthocyanidin (pathway C in Fig. 2), it is unlikely that ANS is capable of catalyzing the dehydration of flavan-2,3,4-triol, because it has a similar structure to that of leucoanthocyanidin. In addition, currently there is no report that describes an additional dehydratase activity by 2-oxoglutarate-dependent oxygenases, although ANS may act to promote the dehydration as a general acid-base catalyst due to the common nature of proteins. The mechanism involving C-3 hydroxylation (iii) may be more plausible in analogy to the reaction catalyzed by flavanone 3-hydroxylase. To elucidate the detailed mechanism of 3-flaven-2,3-diol formation, further investigation may be required.

Fig. 8 illustrates the most probable reaction mechanism from leucoanthocyanidin (flavan-3,4-diol) to the colored anthocyanidin 3-glucoside (flavylium ion) obtained in this study. The reaction catalyzed by 2-oxoglutarate-dependent oxygenases has been postulated to proceed in two steps (37–40). In the first step, ANS binds with ferrous ion, which acts as a catalytic center of the reaction, and composes a complex with molecular oxygen and 2-oxoglutarate, followed by the formation of an oxoferryl enzyme complex, succinate, and CO₂. This first step is common to all 2-oxoglutarate-dependent oxygenases. In the second step, the oxoferryl species is used for the hydrogen radical abstraction from C-2 and C-3 or hydroxylation at C-2 or C-3, followed by spontaneous or, less likely, ANS-catalyzed dehydration that yields 2-flaven-3,4-diol and H₂O. Subsequently, isomerization of the hydroxyl group and double bond of 2-flaven-3,4-diol occurs spontaneously to yield the thermodynamically more stable 3-flaven-2,3-diol (pseudobase) (41) at cytosolic pH. The 3-GT catalyzes 3-O-glucosylation of the pseudobase form under cytosolic neutral conditions. The pseudobase form of anthocyanidin 3-glucoside is then transported into vacuoles, and under the moderate acidic condition of vacuoles (pH 5), the colored flavylum ion of anthocyanidin 3-glucoside is formed by removal of the hydroxyl anion from the C-2 position of the pseudobase form. For the net formation of colored anthocyanidin 3-glucoside (flavylium ion form) from leucoanthocyanidin, only two enzymes, ANS and 3-GT, are therefore required, and the change of pH following transport of anthocyanidin 3-glucoside into vacuoles is sufficient and essential for the coloring of anthocyanidin 3-glucoside (or more modified anthocyanins).
It has been suggested that ANS and 3-GT might work as a multienzyme complex (2). If an ANS-3-GT complex were formed to catalyze the sequential reactions, the intermediate of the reactions, anthocyanin, could then be rapidly channeled between these two enzymes. However, no evidence has been provided for the channelling of the intermediate (Fig. 6). In addition, our recent study with yeast two-hybrid experiments suggests that there is no apparent interaction between ANS and 3-GT from P. frutescens.3 Because a protein-protein interaction between several anthocyanin biosynthetic enzymes of Arabidopsis has been reported recently (42), a more complicated organization of the entire biosynthetic machinery may be involved.

Acknowledgments—We thank Dr. C. Martin, Dr. A. Gierl, and Dr. H. Saedler for the supply of cDNA clones. We also thank Dr. P. Jones (in our laboratory) for suggestions on English usage in the manuscript.

REFERENCES
1. Heller, W., and Forkmann, G. (1988) in The Flavonoids: Advances in Research Since 1980 (Harborne, J. B., ed) pp. 399–425, Chapman & Hall, London.
2. Heller, W., and Forkmann, G. (1993) in The Flavonoids: Advances in Research Since 1986 (Harborne, J. B., ed) pp. 499–535, Chapman & Hall, London.
3. Forkmann, G., and Heller, W. (1999) in Comprehensive Natural Products Chemistry (Sankawa, U., ed) Vol. 1 (Polyketides and Other Secondary Metabolites including Fatty Acids and Their Derivatives) pp. 713–748, Elsevier Science Publishers B.V., Amsterdam.
4. Menssen, A., Höhnmann, S., Martin, W., Schnable, P. S., Peterson, P. A., Saedler, H., and Gierl, A. (1990) EMBO J. 9, 3051–3057.
5. Martin, C., Prescott, A., Mackay, S., Bartlett, J., and Vrijlandt, E. (1991) Plant J. 1, 37–49.
6. Weiss, D., van der Luit, A. H., Kroon, J. T. M., Mol, J. N. M., and Kooter, J. M. (1993) Plant Mol. Biol. 22, 893–897.
7. Davies, K. M. (1993) Plant Physiol. 103, 1015.
8. Sparrell, F., Martin, C., Scienza, A., Gavazzi, G., and Tonelli, C. (1994) Plant Mol. Biol. 24, 743–755.
9. Pelletier, M. K., Murrell, J. R., and Shirley, B. W. (1997) Plant Physiol. 113, 1437–1445.
10. Saito, K., Kobayashi, M., Gong, Z., Tanaka, Y., and Yamazaki, M. (1999) Plant J. 17, 181–189.

3 K. Saito et al., unpublished observations.
