Structure of commelinin, a blue complex pigment from the blue flowers of *Commelina communis*

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Abstract: The X-ray crystal structure of natural commelinin is investigated. The results demonstrate that commelinin is a tetranuclear (4 Mg^{2+}) metal complex, in which two Mg^{2+} ions chelate to six anthocyanin molecules, while the other two Mg^{2+} ions bind to six flavone molecules, stabilizing the commelinin complex, a new type of supramolecular complex.

Keywords: commelinin, metal complex, blue pigment, anthocyanin, X-ray crystal structure

Introduction

Anthocyanin pigments responsible for flower colors, from red to blue,[1),2)] are generally isolated as red oxonium salts using acid solvents. However, isolation of the native pigments without changing colors and their characterization would be indispensable to clarify the mechanism of flower color variation. Commelinin, a blue pigment from the blue flowers of *Commelina communis* L., was first isolated in 1957 using neutral solvents[3)] and was shown to be a complex composed of a reddish purple anthocyanin, a pale yellow flavone and magnesium.[4)–6)] Bluing by chelation of a bivalent metal ion, the Mg^{2+} ion, with anthocyanin was regarded as suspicious,[7)–9)] however, reconstruction of commelinin and metal-substituted commelinins using the components demonstrated that a Mg^{2+} ion is essential.[10)–12)] X-ray analysis using reconstructed Cd-commelinin[11)] indicated that commelinin was a binuclear (2 Mg^{2+}) metal complex containing anthocyanin and flavone.[13),14)] However, the real features of natural commelinin still remained obscure, because of difficulty of preparation of the crystals needed for X-ray analysis.[15)] Recently we succeeded in the preparation of single crystals of natural commelinin. Here we report its X-ray crystal structure.

Material and methods

Natural commelinin was isolated from the fresh petals of *Commelina communis* L. var. *hortensis* Makino (Fig. 1a) principally in the same way as reported previously.[4)–6)] Press juice (3.1 l) from the fresh petals (3.8 kg) was added with 6 vols. of ethanol, whereby an amorphous blue pigment (ca 5.5 g) was obtained. The pigment was further purified by Sephadex (LH 20) column chromatography (2.5 × 35 cm) using water as an eluant and repeated fractional precipitation from aqueous ethanol solutions. The blue pigment was separated from aqueous ethanol solutions in the form of rectangular prisms. Recrystallization was repeated more than three times at 5 °C. Finally, single crystals available for X-ray analysis were separated. Preparation of crystals of reconstructed and metal substituted commelinins was carried out with the same methods described previously,[10),11)] using purified anthocyanin (AN) (malonylawobanin,[16)] delphinidin 3-*O*-(6-*O*-*p*-coumaroylglucoside)-5-*O*-(6-*O*-malonylglucoside), Fig. 1b) and flavone (FL) (flavocommelin,[17),18)] swertisin 4′-*O*-glucoside, Fig. 1b).

All the crystals were sealed in glass capillaries for diffraction measurements and data sets were collected on BL-6A at the Photon Factory in KEK, using an ADSC Quantum-4R CCD detector. The data sets were processed with the HKL2000 package.[19)] All the crystals belong to space group *P*321

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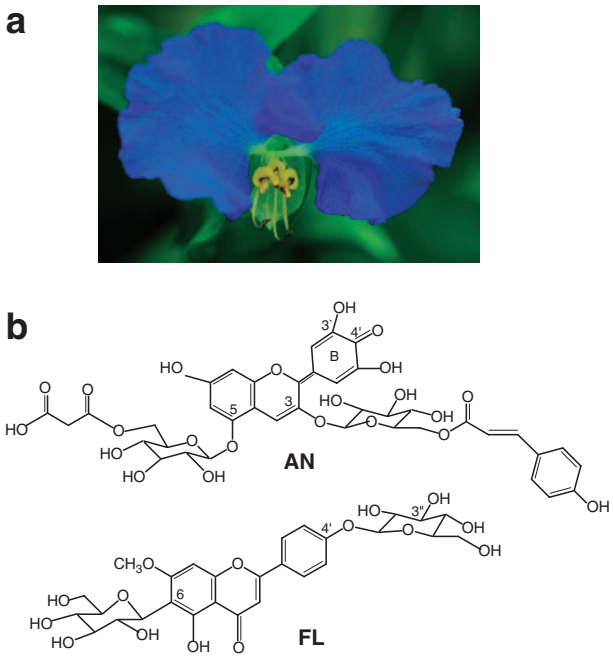


Fig. 1. A blue flower of *Commelina communis* and organic components of commelinin. **a**, A blue flower of *Commelina communis* L. var. *hortensis* Makino. **b**, The components of commelinin, anthocyanin (AN): Malonylawobanin [delphinidin 3-*O*-(6-*O*-*p*-coumaroylglucoside)-5-*O*-(6-*O*-malonylglucoside)] and flavone glycoside (FL): Flavocommelin [swertisin 4′-*O*-glucoside].

where an asymmetric unit contains one third of a molecule (two ANs and two FLs). The initial phase sets were obtained by an *ab initio* density modification method using program LODEM[20),21)] and the structures were refined with SHELXL97.[22)] The final R-factor for the natural commelinin crystal was 0.1124 for 11786 reflections ($> 4\sigma(F)$). Figures were prepared using the program UCSF Chimera.[23)]

Results and discussion

The crystal structure of natural commelinin was determined at 0.95 Å resolution. The refined molecule has a three fold symmetry and four Mg^{2+} ions (Mg1∼Mg4) align along a three-fold axis (Fig. 2a, b, Fig. 3a, b). The four metals are coordinated to six AN molecules and six FL molecules, forming an ellipsoidal shape with a dimension of approximately 30 Å × 30 Å × 20 Å (Fig. 2a, b). The distances among the four Mg^{2+} ions are Mg4-Mg1, 9.512 Å; Mg1-Mg2, 4.940 Å and Mg2-Mg4, 6.781 Å respectively (Fig. 3a, b).

The inner two Mg^{2+} ions, Mg1 and Mg2 are coordinated to the oxygen atoms of position 3′ and 4′ of the delphinidin nucleus of AN, in bond distances of Mg1-O (3′), 2.088 Å; Mg1-O (4′), 2.087 Å and Mg2-O (3′) 2.091 Å; Mg2-O (4′) 2.092 Å, respectively (Fig. 2b, Fig. 3a, 3c). On the other hand, Mg3 binds to the oxygen atom of position 3″ of glucose attached to position 4′ of the swertisin nucleus of FL, in bond length of 2.115 Å and three water molecules (W1) on the outer surface with the average distance of 2.031 Å (Fig. 2b, Fig. 3b). Mg4 binds to the oxygen atom of position 3″ (O3) of glucose attached to the 4′ position of swertisin via a water molecule (W2) and to three water molecules (W3) (Fig. 2b, 3b). The bond lengths of Mg4-H_2O(W2)-O(3) are 2.056 Å and 2.715 Å, and that of Mg4-H_2O (W3) is 2.043 Å. The temperature factors of the water molecules (W2 and W3) as well as Mg4 are small, and thus, the contribution of the water molecules to the Mg4 and O(3) interaction is assumed to be significant.

In the commelinin molecule, both AN and FL are self-associated with each other as AN-AN and FL-FL in pairs. The distances between the aromatic rings of each AN-AN and FL-FL are both approximately 3.3 Å, indicating hydrophobic interaction (Fig. 3a, b). The inner Mg1 and Mg2 ions are each coordinated to a different AN fragment of the associated AN-AN pair (Fig. 3a). Accordingly each of the two Mg^{2+} ions binds with three ANs, one from each of the three AN-AN pairs, forming a three-bladed propeller-like arrangement (Fig. 4a). On the other hand, the outer two Mg^{2+} ions, Mg3 and Mg4, are each coordinated to a separate FL of the associated FL-FL pair (Fig. 3b) and consequently two Mg^{2+} ions each bind to three FLs of the associated FL-FL pairs in a three-bladed propeller-like arrangement (Fig. 4b and 4c). In addition, Mg3 and Mg4 coordinate to three water molecules forming an octahedral geometry respectively (Fig. 4d and 4e).

The bond distances of the B-ring of AN in the commelinin molecule are shown in Fig. 3c. These data apparently indicate that the B-ring of AN is in 4′-keto-quinoidal form, and thus, AN chelates with the Mg^{2+} ion in the form of a 4′-keto-quinoidal base anion.

Commelinin was hitherto known as a binuclear complex,[13),14),24)–26)] however, our results indicate that natural commelinin is a tetra-metal nuclear

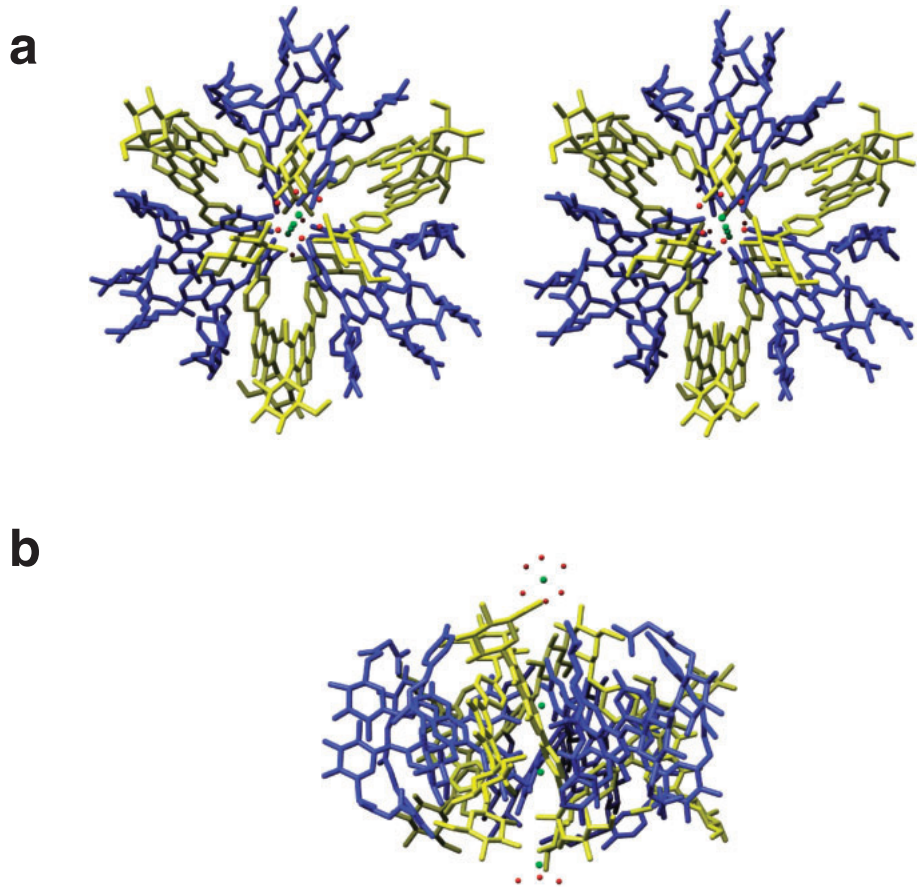


Fig. 2. Crystal structure of the commelinin molecule. Blue, anthocyanin (AN); yellow, flavone glycoside (FL); green balls, Mg^{2+} ions; red balls, water molecules. **a**, Stereo view along the three-fold axis. **b**, A side view.

complex. To confirm the total structure of commelinin, we reconstructed commelinin, Mg-commelinin and metal-substituted commelinin, Cd-commelinin, using purified AN and FL and metals in a manner similar to that described previously,[10),11)] and further investigated by X-ray crystallography. The results indicate that X-ray crystal structures of the reconstructed Mg- and Cd-commelinins are basically the same as that of natural commelinin.

Commelinin is known to be a stable blue complex pigment even in acidic condition.[4),5),12)] Natural commelinin showed a blue color and exhibited two characteristic absorption maxima in the visible region, 589.1 nm and 644.1 nm, even in 10 mM acetic acid (pH 3.30) and also in 20 mM potassium acetate buffer of pH 3.80. The spectra were almost the same as those in water and acetate buffer of pH 5.80. Copigmentation, the anthocyanin-copigment complex, is generally affected by pH.[27)] In fact, AN-FL (1:1) mixtures gave a purple color at pH 5.80, having an absorption maximum at 542.5 nm and a shoulder at 572.5 nm in the visible region, while the color changed to purplish red (λ_{max} 536.5 nm) in acetate buffer of pH 3.80 and to red (λ_{max} 534.3 nm) in 20 mM acetic acid (pH 3.30). We prepared the crystals of natural commelinin from the solutions of 20 mM acetate buffer pH 3.80 and 10 mM acetic acid pH 3.30, by adding ethanol respectively. X-ray structures of both samples were practically the same as that described above and four Mg^{2+} ions, Mg1∼Mg4, remained in the complex molecule. Furthermore, the occupation level of the outer two Mg^{2+} ions, Mg3 and Mg4, as well as the inner two Mg^{2+}, Mg1 and Mg2, was not decreased. The results indicate that the total structure of the tetra-metal nuclear complex of commelinin remains constant even in acidic conditions.

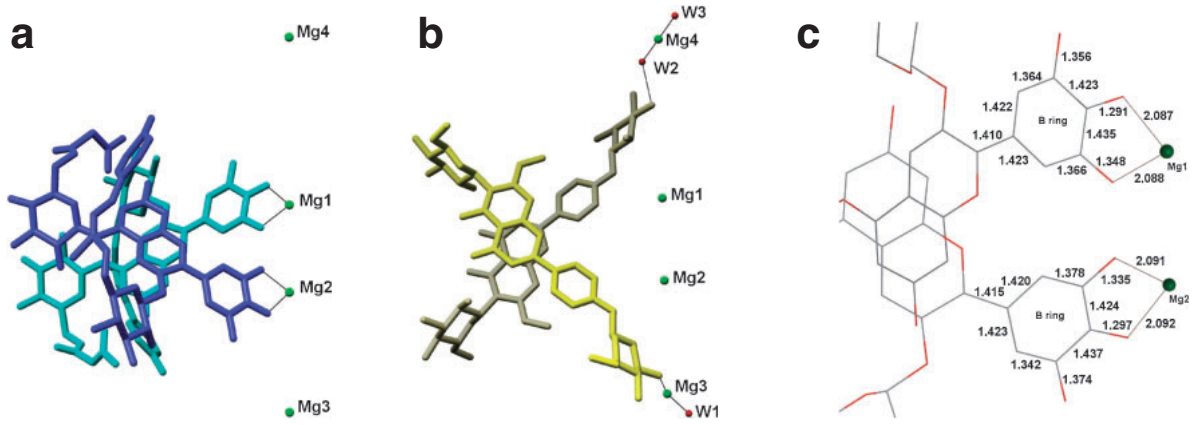


Fig. 3. Metal binding sites in the commelinin molecule. Green balls, Mg^{2+} ions; red balls, water molecules. **a**, A side view of stacking AN and AN. One AN binds to an inner Mg^{2+} ion, Mg1, while the other binds to another inner Mg^{2+} ion, Mg2. Blue, the front side AN; cyan, back side AN. **b**, A side view of stacking FL and FL. One FL binds to an outer Mg^{2+} ion, Mg3, while the other binds to another outer Mg^{2+} ion, Mg4, via water molecule, W2. Yellow, the front side FL; khaki, the back side FL. **c**, The bond distances (Å) of the B-rings of ANs in the commelinin molecule. Green balls, Mg^{2+} ions.

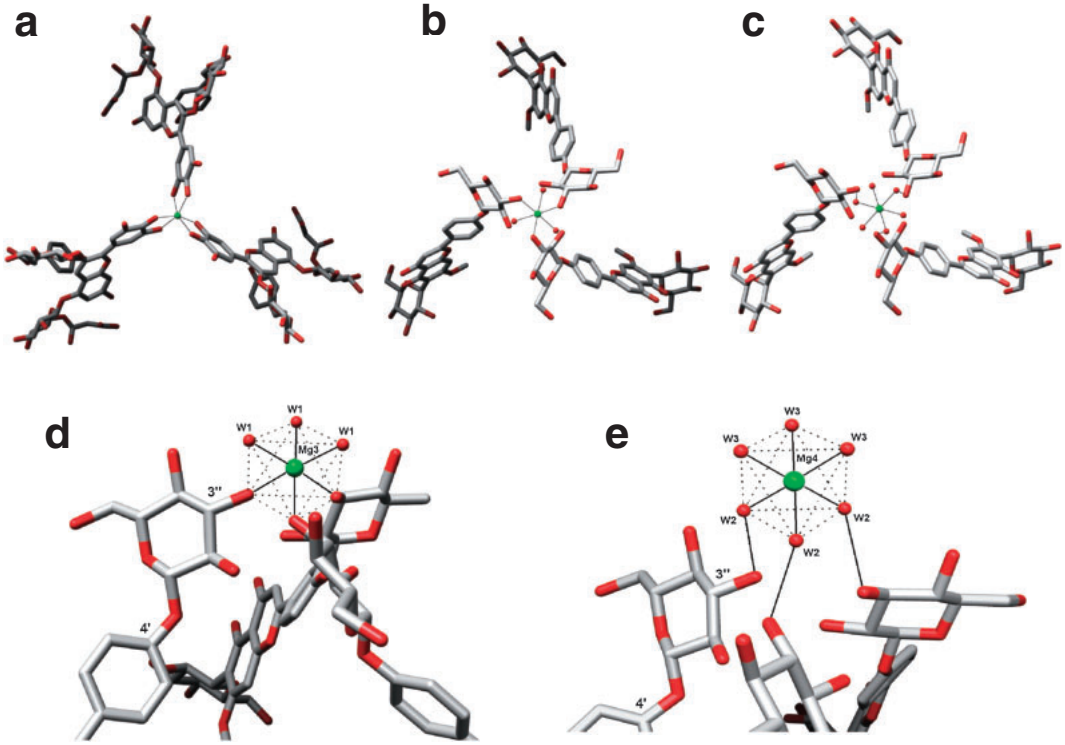


Fig. 4. Metal binding sites in the commelinin molecule. Green balls, Mg^{2+} ions; red balls, water molecules. **a**, A view along the three-fold axis of an inner Mg^{2+} ion (Mg1 or Mg2) site. The bicolor frames, ANs. **b**, A view along the three fold axis of the outer Mg3 site. The bicolor frames, FLs. **c**, A view along the three fold axis of the outer Mg4 site. The bicolor frames, FLs. **d**, Mg3 binding site. Mg3 binds to the oxygen atom of position 3″ of glucose attached to position 4′ of the swertisin nucleus of FL and three water molecules (W1) on the outer surface, forming octahedral geometry as shown by dotted line. **e**, Mg4 binding site. Mg4 binds to the oxygen atom of position 3″ of glucose attached to the 4′ position of swertisin via water molecule (W2) and to three water molecules (W3), forming octahedral geometry as shown by dotted line.

The present work revealed the molecular structure of natural commelinin by X-ray crystallography and demonstrated that the blue color is developed by a homometallic tetra-nuclear complex pigment comprising six AN molecules, six FL molecules and four Mg^{2+} ions. The arrangement of anthocyanins, flavone glycosides and metal ions in commelinin is similar to that in protocyanin, a blue complex pigment from *Centaurea cyanus*, which is a heterometallic tetra-nuclear (Fe^{3+}, Mg^{2+}, $2Ca^{2+}$) complex pigment.[28),29)] In the commelinin molecule, the chelate formation of two Mg^{2+} ions with the 4′-keto-quinoidal base of AN plays an important role for the bluing and coordination of the other two Mg^{2+} ions to FL has a crucial role for giving stability to the complex molecule.

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