Anthocyanin Intramolecular Interactions. A New Mathematical Approach To Account for the Remarkable Colorant Properties of the Pigments Extracted from *Matthiola incana*

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Abstract: In the last few years, a series of investigations has brought to light a mechanism of stabilization of the colorant properties of certain anthocyanins. Intramolecular interactions take place between the chromophore moiety of the anthocyanin and one of its aromatic acid residues, which folds over the chromophore and thus confers protection against hydration and subsequent formation of colorless forms. In our continuing study of the physicochemical properties exhibited by acylated natural anthocyanins, we report here on a series of five structurally related pigments extracted from the violet flowers of *Matthiola incana*. These pigments all bear the same chromophore moiety, i.e., the cyanidin aglycon, but differ in the degree of glycosylation and acylation. Acidity constants for the deprotonation of the cyanidin aglycon are determined from UV-visible absorption measurements. The data support the existence of intramolecular, noncovalent interactions that strongly stabilize the colored forms of the pigments. However, none of the four more heavily substituted anthocyanins follows the above-mentioned mechanism that was previously successfully applied to the study of acylated anthocyanins. Consequently, a new mechanism with a different mathematical treatment is here developed to account for the different behavior exhibited by these distinctive pigments.

Introduction

Anthocyanins, glycosylated polyhydroxy derivatives of 2-phenylbenzopyrylium (flavylium) salts, are the pigments responsible for most of the wide variety of colors displayed by flowers and fruits, where they are naturally produced. However, when extracted from plant material, the most common anthocyanins (3-monoglucosides and 3,5-diglucosides) are almost colorless in mildly acidic equilibrated aqueous solution. However, when extracted from plant material, the most common anthocyanins (3-monoglucosides and 3,5-diglucosides) are almost colorless in mildly acidic equilibrated aqueous solution, whereas pigments extracted from plant cells. This color loss is due to an hydration reaction (Scheme 1) of the colored flavylium cation, which produces the colorless forms hemiacetal and chalcones.

In order to explain the remarkable color stability of anthocyanins in nature, several studies were performed in model solutions containing the pigments and some natural colorless molecules, currently known as copigments. The results obtained, using UV-visible and ¹H NMR spectrometries, evidenced the existence of an intermolecular noncovalent interaction (copigmentation) between the anthocyanin and

Scheme 1

![Scheme 1](image-url)
copigment molecules. Such hydrophobic complexation efficiently protects the chromophore against the nucleophilic water attack, thus displacing the equilibria of Scheme 1 toward the formation of more flavilyum molecules.

Since the last decade, with the improvement of extraction and purification techniques, far more complex anthocyanin structures, which gave stable colored solutions at mildly acidic 

\[ \text{AH}^+ + \text{H}_2\text{O} \rightleftharpoons (\text{B} + \text{C}_d) + \text{H}^+ \]  

(2)

Since the ring opening of B to form the E-chalcone occurs in a very fast step,\(^\text{18,19}\) we write the equilibrium as a whole for the sake of clarity; \(\text{C}_d\), which is usually formed in very small amounts,\(^\text{20}\) is neglected. All the acylated molecules studied until now present a decrease in the value of \(K_h\),\(^\text{13–15}\) and only minimal changes in the deprotonation constant \(K_a\), which signifies that the interaction between the two moieties of the pigment, while in the flavilyum cation form, proceeds through hydrophobic stacking and not through hydrogen bonding, since the existence of free OH groups in the aglycon is a requisite for formation of the quinonoidal bases, A (Scheme 1).

The present work reports on the first known group of acylated anthocyanins that represents a deviation to this, until now, general behavior. The five pigments, which constitute a structurally homogenous series based on the cyanidin (3,5,7,3′,4′-pentahydroxyflavylium) aglycon, were extracted from the violet flowers of *Matthiola incana*.\(^\text{21}\) Their structures (Chart 1), fully elucidated by 1H NMR techniques and FAB-MS, are 3-O-(2-O-β-D-xylopyranosyl-β-D-glucopyranosyl)-5-O-β-D-glucopyranosyl cyanidin (1), 3-O-(6-O-(trans-feruloyl)-2-O-(2-O-(trans-sinapyl))-β-D-glucopyranosyl)-5-O-β-D-glucopyranosyl cyanidin (2), 3-O-(6-O-(trans-p-coumaryl)-2-O-(2-O-(trans-sinapyl))-β-D-glucopyranosyl)-5-O-(6-O-(malonyl)-β-D-glucopyranosyl cyanidin (3), 3-O-(6-O-(trans-cafeoyl)-2-O-(2-O-(trans-sinapyl))-β-D-glucopyranosyl)-5-O-(6-O-(6-O-(malonyl)-β-D-glucopyranosyl cyanidin (4), and 3-O-(6-O-(trans-feruloyl)-2-O-(2-O-(trans-sinapyl))-β-D-glucopyranosyl)-5-O-(6-O-(malonyl)-β-D-glucopyranosyl cyanidin (5). The thermodynamic and kinetic data gathered are compared to the ones obtained for cyanidin 3,5-diglucoside (cyanin), one of the most common anthocyanins that can be considered a simpler structural analogue, and reveal not only a marked increase in the value of \(K_{\text{a}}\) for the more complex pigments but also an otherwise unknown effect of acidity-dependent interaction, which prompted us to discuss the results obtained in the light of a new mechanistic and mathematical approach.

**Experimental Section**

**Materials.** Pigments 1–5 were isolated from the violet flowers of *Matthiola incana* according to a published procedure.\(^\text{21}\) Their purity, always superior to 98%, was checked by 1H NMR spectroscopy. Cyanidin 3,5-diglucoside, henceforward referred to as cyanin, was a kind gift of Prof. Sam Asen and was used without further purification, and

\begin{itemize}
\end{itemize}
Table 1. Maximum Visible Wavelength and Molar Absorption Coefficient for the Flavylium Cation Form of the Seven Anthocyanins

<table>
<thead>
<tr>
<th>pigment</th>
<th>cyanin</th>
<th>antirrhinin</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
</tr>
</thead>
<tbody>
<tr>
<td>( \lambda_{\text{max}} / \text{nm} )</td>
<td>508</td>
<td>35 000(^{32} )</td>
<td>510</td>
<td>7000</td>
<td>522</td>
<td>528</td>
<td>536</td>
</tr>
<tr>
<td>( \epsilon_{\text{m}} / \text{mol}^{-1} \text{ dm}^{3} \text{ cm}^{-1} )</td>
<td>3600</td>
<td>10 000</td>
<td>15 100</td>
<td>19 000</td>
<td>21 200</td>
<td>20 100</td>
<td></td>
</tr>
</tbody>
</table>

* pH = 0.9.

here reported, there is one (pigment 1) where the hypochromic effect is far more stronger, exhibiting an \( \epsilon \) ca. ten times smaller than that published for cyanin\(^{2} \) (Table 1). Such a large drop in the value of the absorption coefficient seems to be a general characteristic of the molecules possessing a disaccharide as a substituent group in position 3 of the chromophore, since a similar pattern was already noticed in two other anthocyanins presenting an analogous substitution pattern (delphinidin 3-gentiobioside and antirrhinin).\(^{15,22} \) Although no other values for molecules with a structure similar to 1 are of our knowledge, this effect seems to be more intense when there is also a glycosyl residue in position 5, when compared with 1 with antirrhinin, which shares the same chromophore, the first shows an \( \epsilon \) value of roughly 50%\(^{25} \) that of the latter (Table 1).
Figure 2. Electronic absorption spectra of 3 (1.8 × 10⁻⁵ M, T = 25 °C) in aqueous solution, before (a) and after (b) the subtraction of the flavylum cation absorption. 1, pH 0.8; 2, pH 1.6; 3, pH 2.0; 4, pH 2.8; 5, pH 3.5.

the, until now, generally observed formation of this form at a pH closer to neutrality.²

The observation of these distinctive characteristics for anthocyanins 2–5, together with the unapplicability of the model postulated in ref 14 led us to bring forth the following hypothesis of mechanism to account for the behavior of the four pigments as a function of pH, in acidic aqueous solutions.

$$\text{AH}^+ + K_1 \rightleftharpoons \text{CP}$$

$$\text{CP} + H_2O \rightleftharpoons K_{CP} \rightleftharpoons B + H^+$$

$$\text{CP} \rightleftharpoons K_{CP} \rightleftharpoons A + H^+$$

with $$K_1 = [\text{CP}][\text{AH}^+]$$, $$K_{CP} = [\text{B}][\text{H}^+][\text{CP}]$$, and $$K_{CP} = [\text{A}][\text{H}^+][\text{CP}]$$. In eq 4 the fast equilibrating B and C₆ forms are simply represented by B for the sake of clarity. Equation 3 accounts for the assumed equilibrium between two conformations of the flavylum cation, one with an acyl residue folded over the chromophore in the form of an intramolecular complex (CP), with a smaller $$\epsilon$$ value than that of the other conformation (AH⁺) which is not copigmented. AH⁺ has its domain of existence only in very acidic media (pH < 1), while CP dominates in the pH region 1.0–2.0. This would explain the sudden drop of absorbance verified when going from very acidic to slightly less acidic solutions. It will be the intramolecular copigmentation complex thus formed that undergoes the usual reactions—eqs 4 and 5—of hydration and deprotonation depicted in Scheme 1.

Equations 6–8 represent the overall concentration of pigment (C₇), the visible absorbance of an anthocyanin solution for a given pH (Dₐ), and at pH < 1 (D₀), where only AH⁺ absorbs

$$C_T = [\text{AH}^+] + [\text{CP}] + [\text{A}] + [\text{B}]$$

$$D = \epsilon_{\text{AH}}[\text{AH}^+] + \epsilon_{\text{CP}}[\text{CP}] + \epsilon_A[\text{A}]$$

A plot of D₀/D as a function of 1/[H⁺] for pigment 5, according to eq 10.

**Table 2.** Thermodynamic Parameters Obtained from Eq 10 for Pigments 2–5

<table>
<thead>
<tr>
<th>pigment</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
</tr>
</thead>
<tbody>
<tr>
<td>K₁</td>
<td>1.98</td>
<td>1.91</td>
<td>1.94</td>
<td>1.94</td>
</tr>
<tr>
<td>k¹</td>
<td>8.20</td>
<td>8.77</td>
<td>8.20</td>
<td>8.20</td>
</tr>
<tr>
<td>R</td>
<td>0.998</td>
<td>0.998</td>
<td>0.998</td>
<td>0.999</td>
</tr>
</tbody>
</table>

*) T = 25 °C. b Pigment 2 gives a K₁ = 1.15; see text for discussion.

Kinetic Data. The pH jumps conducted at constant temperature for all the pigments studied produce, without exception, an exponential decay of the visible absorbance, caused by the pH-dependent hydration reaction. However, the ensemble of kinetic data gathered, like the thermodynamic ones, should be analyzed according to two different models. Thus, cyanin, antirrhinin, and I follow the treatment, presented elsewhere,¹⁴ which uses eq 11 to fit the data gained from the spectrophotometric analysis.

$$(K_a + K_h + [H^+]) = \frac{1}{k} + \frac{1}{[H^+]k_2}$$

In eq 11, $$k_2$$ represents the dehydrogenase rate constant as defined by the ratio $$K_h = k_h/k_2$$, where $$k_h$$ defines the rate constant for the direct process (hydration). By plotting $$(K_a + K_h + [H^+])/k$$ vs 1/[H⁺], one can obtain directly $$k_2$$ and $$K_h$$, and, together with the values obtained from the plot of D₀/D vs [H⁺], above mentioned, also the values for Kₐ and k₁.

For the remaining pigments (2–5) it is necessary to take into account the reaction scheme proposed in eqs 3–5, which leads...
to the following treatment for the experimental data obtained through the spectrophotometric measurements.

Equation 12 describes the relaxation process of the hydration equilibrium after a pH-jump

\[
\frac{d[B]}{dt} = k_{CP}^{CP}[CP] - k_{2}^{CP}[B][H^+] \tag{12}
\]

with \( k_{1}^{CP} = k_{2}^{CP}/k_{2}^{CP} \). If the principle of mass conservation is expressed as

\[
\Delta[AH^+] + \Delta[CP] + \Delta[A] + \Delta[B] = 0 \tag{13}
\]

one can combine eqs 12 and 13 to obtain the apparent first-order rate constant \( k_{CP} \) as defined by \( -d[A]/dt = -k_{CP}[A] \). It comes in the form

\[
k_{CP} = k_{2}^{CP}[H^+] \left( \frac{k_{a}^{CP} + k_{h}^{CP} + [H^+](1 + \frac{1}{K_{1}})}{k_{a}^{CP} + [H^+](1 + \frac{1}{K_{1}})} \right) \tag{14}
\]

Equation 14 can be written in the following way

\[
\frac{k_{a}^{CP} + k_{h}^{CP} + [H^+](1 + \frac{1}{K_{1}})}{k_{CP}^{2}} = 1 + \frac{1}{K_{1}} + \frac{k_{a}^{CP} + k_{h}^{CP}}{k_{a}^{CP} + [H^+](1 + \frac{1}{K_{1}})} \tag{15}
\]

Thus, a plot of \( \{k_{a}^{CP} + k_{h}^{CP} + [H^+](1 + \frac{1}{K_{1}})\}/k_{CP}^{2} \) as a function of \( 1/[H^+] \) yields a straight line with an intercept of \( 1 + 1/K_{1}/k_{2}^{CP} \), from where the value of \( k_{2}^{CP} \) can be readily obtained if one takes the value for \( K_{1} \) obtained from eq 10, and a slope equal to \( k_{a}^{CP}/k_{2}^{CP} \), which leads immediately to the values for \( K_{CP}^{2}, K_{CP}^{3}, \) and \( k_{1}^{CP} \). The values obtained for the thermodynamic and kinetic constants of all the pigments studied are presented in Table 3.

**Discussion**

Water, in its liquid state, is generally considered as a structured network of hydrogen-bonded molecules\(^{27-29}\) responsible for the specificity of most biological processes. It is also known that even minor physical or chemical changes can disrupt this particular three-dimensional organization and thus inactivate these processes.\(^{28}\) Intermolecular copigmentation, a phenomenon driven by hydrophobic interactions, is likewise dependent on solvent arrangement, being more strong at temperatures near 0 °C than at room temperature, since at the former water is a quasi-crystalline medium which shows favor to the interaction between anthocyanin and copigment.\(^{67}\) Given this reasoning, it is possible to think of the “pH dependent” intramolecular copigmentation of the flavylium cation form of anthocyanins \( \text{2} - \text{5} \), as associated to a change in the particular framework of liquid water caused by the increase in acidity which will give rise to a less organized solvent structure, at very acidic pH values, thus hindering the folding of the acyl residue over the chromophore.

From the analysis of the data presented in Table 2, it is evident a common value of \( K_{1} \approx 8.0 - 9.0 \) for pigments \( \text{3} \) and \( \text{4} \), and \( \text{5} \) seems to justify the mechanism conjectured above, eqs \( \text{3} - \text{5} \). Pigment \( \text{2} \) has a \( K_{1} \), and a spectrophotometric behavior intermediate between what is typically reported for other anthocyanins and the spectral characteristics of compounds \( \text{3} - \text{5} \). Nevertheless, the theoretical interpretation of the absorption gain \( \text{vs} \) pH, for this molecule, is better achieved through the application of eq 10 (\( R = 0.9986 \)) than by the use of the formalism reported in refs 13 - 15 (\( R = 0.9920 \)). This predicts an important role for malonyl residues on the formation of intramolecular complexes by anthocyanins, since this is the sole difference between pigments \( \text{2} \) and \( \text{5} \) (\( \text{3} \) and \( \text{4} \) are also structurally closer to \( \text{5} \)). The influence of the malonyl group is already manifest when comparing the spectra of the flavylium cations of \( \text{2} \) and \( \text{5} \) (Figure 4), notably the band at ca. 320 nm, characteristic of the acylated anthocyanins, which is less intense than the one at 280 nm for \( \text{2} \), while the inverse happens with \( \text{3}, \text{4}, \) and \( \text{5} \). Such increase in band intensity could be used as an empirical tool for the characterization of simultaneously acylated and malonylated anthocyanins.

The data reported in Table 3 allows the following assumptions: (i) The well-known increase in \( pK_{a} \) when going from 3,5-diglucosylated molecules to 3-glucosylated ones,\(^{15,30}\) can also be extended to antirrhinin (relatively to cyanin), which possesses a particular type of disaccharide (a rutinose) bound to position 3. (ii) A very low \( pK_{a} \) value for \( \text{1} \), which can now be considered as a distinguishing feature of some types of 3-disaccharides, which possess the ability of preferential stabilization of the hemiacetal form by formation of intramolecular hydrogen bonds between the hemiacetal OH and one of the free hydroxyls of the terminal sugar.\(^{15}\) Molecular mechanics calculations, performed in a simulated aqueous solution, with several possible structural conformations, yielded for the conformation with the lower enthalpy value, a distance of 3.18 Å between the above two hydroxyl groups, which is generally considered as being capable of accommodating an hydrogen bond.\(^{27-29}\) Similar calculations, performed with antirrhinin, showed no such behavior, this being attributed to

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Although the presence of a terminal CH₃ group, instead of an hydroxyl, in the sugar position favorable for the formation of an H bond. (iii) The pKₐ values in the order 3, 2, 5, and 4. Although k₋CP values increase in the order 2, 5, and 4. This behavior can be interpreted through the analysis of the data collected from molecular modelization of the four pigments, and the comparison of the structures with the minimum relative enthalpies obtained for each compound. These results evidence the existence, for all those four anthocyanins, of a “sandwich” structure with the two aromatic acyl residues positioned on each side of the chromophore, thus protecting it from hydration, the difference in protection being given by the relative position of the acid ring I relative to the B-ring of the aglycon, the closer it is the lesser the efficiency of the nucleophilic attack. This closeness also increases in the order 3, 2, 5, and 4. This increase in vicinity of the two aromatic rings is accompanied, and presumably caused, by an increasing proximity between the final carbonyl group of the malonyl residue and the 7-OH of the aglycon. (iv) Since the hydroxyl groups in positions 4 and 7 are the ones that participate in the deprotonation equilibria to form the quinonoidal bases, the relative proximity of the malonyl to the 7-OH, with the possibility of H bond formation, should increase the charge density in C-7 and thus facilitate the deprotonation, with the concomitant decrease in pKₐ . This assumption is further stressed by the inspection of the values computed for the above mentioned interatomic distances, which are respectively 3.43 Å for pigment 4, 3.60 Å for 5, and 4.72 for 3, following the increase in pKₐ . Since molecule 2 does not possess a malonyl residue, its pKₐ is higher than the ones calculated for the above three pigments, although lower than the ones obtained for cyanin or 1. This peculiar interaction reveals an important role for malonyl residues in natural colorants, since this lowering of the pKₐ , together with the protection against color loss by hydration, allows the existence of colored forms (flavylum cation or quinonoidal base) through a very large range of acidic pH values. The cornerstone of the mechanism of color stabilization and variation in the case of this series of pigments rests on the formation of the quinonoidal bases at low pH values. This is true for instance in the case of compound 3 which has a pKₐ value lower than the one found for cyanin. However, the loss of color stability, related to the lowering of the pKₐ value, is, in that case, largely compensated by the decrease in the pK₋CP value when going from cyanin to pigment 3. Finally, it should be noted also the small differences between pK₋CP and pKₐ for pigments 2–5. This is an important characteristic feature of color stabilization and variation within the flowers of the Matthiola incana species.

Conclusions

The formation of intramolecular complexes, which prevent the loss of color through hydration reactions, by natural anthocyanins possessing at least one aromatic acyl residue linked through sugarspacer at the aglycon moiety is here evidenced, especially for compounds 4 and 5, which not only evidence the already known effect of increase in pKₐ but, furthermore constitute, from our knowledge, the two sole natural anthocyanins that present a value for their pKₐ lower than that of the pK₋CP. Moreover, a role for the simple, nonaromatic, malonyl residue, related to this property, is also disclosed for the first time in the present work. A new mathematical treatment of a new equilibrium mechanism, complementary to the one already employed for the characterization of similar pigments, is also presented to account for the particular properties of the set of molecules here discussed.

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