Kinetic and Thermodynamic Control of Flavylium Hydration in the Pelargonidin-Cinnamic Acid Complexation. Origin of the Extraordinary Flower Color Diversity of *Pharbitis nil*

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Abstract: During the past decade, structural elucidation of heavily substituted anthocyanins present, for instance, in the bright ornamental flowers, has brought to light the role played by sugar and phenolic acid residues in the fascinating pigmentation properties of such natural molecules. It now appears that higher plants have developed in their flowers and fruits extremely sensitive and powerful color stabilization and variation mechanisms related to the presence of glycosidic acylated anthocyanidins. In these molecules, a sugar unit, bearing the anthocyanidin chromophore and a cinnamic acid residue, brings sufficient flexibility for the latter two moieties to interact through complexation, according to a mechanism called intramolecular copigmentation. Here, on the basis of UV-visible spectroscopic measurements, we give kinetic and thermodynamic evidence supporting the existence of folded conformations which involve the stacking of either one cinnamic acid residue on the anthocyanidin chromophore or two cinnamic acid residues on both sides of the chromophore (sandwich-type association). Pigments investigated in this work were obtained from red-purple cultivars of *Pharbitis nil* (morning glory). They correspond to four pelargonidin derivatives (**1**–**4**): one which is not acylated (reference compound **1**), two of the monoacylated type (**2** and **3**), and one diacylated (**4**). The cornerstone of our study rests on the hydration reaction of the anthocyanidin chromophore when in its flavylium form. Indeed, the kinetic and thermodynamic parameters of this reaction and the way they are affected by the presence of one or two cinnamic acid residue(s) is of considerable value in the understanding of anthocyanin intramolecular complex formation. Copigmentation more frequently occurs as an intermolecular process, pigment and copigment being in that case two distinct molecules. By running competitive intra- and intermolecular copigmentation experiments, we also demonstrate that the phenomenon in which pigment and copigment are linked together is much more efficient.

Introduction

Copigmentation of anthocyanins (pigments) is one of the most efficient processes providing color stabilization in the plant organs, mainly flowers and fruits, where the anthocyanins occur.[1-5] From UV-visible and ^{1}H NMR investigations dealing with model aqueous solutions containing an anthocyanin and a copigment,[6-10] it has been made clear that copigmentation consists in the π-stacking molecular (noncovalent) interaction of the planar chromophore (anthocyanidin) of the colored forms of the pigment with colorless molecules of copigment. This complexation phenomenon efficiently protects the colored forms from the fading occurring through flavylium ion hydration, leading to the colorless forms, hemiacetal and chalcones (Scheme I). In the absence of copigment and under the physico-chemical conditions prevailing in their natural medium,[11] i.e., in slightly acidic to neutral aqueous solutions, common anthocyanins strongly hydrate and the amount of colored forms usually drops to less than 5% of the total pigment concentration, resulting in almost colorless solutions.[12-14] Thus, the recovery of a deep color from a solution of common anthocyanin requires the presence of an efficient copigment in large excess in order to markedly shift the hydration equilibrium toward the selectively stabilized colored forms.[6,7,15] Therefore, the recent discoveries of anthocyanins, giving deep stable colors at neutral pH in the absence of any copigment, are particularly fascinating.[2,16-34] Modern NMR techniques applied to these

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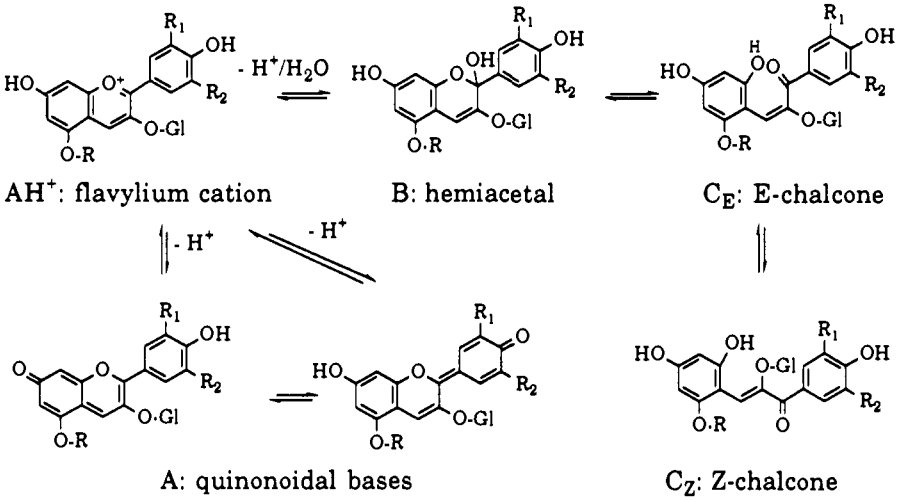
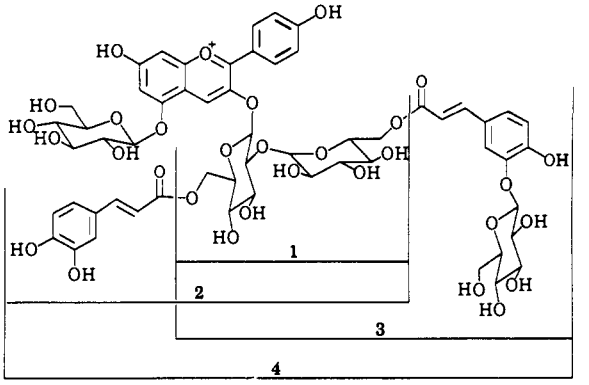
Scheme I. The Structural Transformations of an Anthocyanin in a Slightly Acidic Aqueous Solution[a]



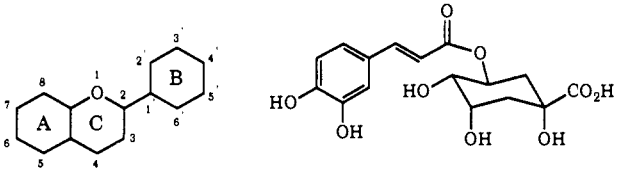
[a] R = H, Gl; R1, R2 = H, OH, OMe, OGl (Gl = glycosyl group).

pigments have allowed a complete elucidation of their structures[18-23,27-30] and have provided reliable pieces of information about their most stable conformations in solution.[27,30] These pigments are acylated anthocyanins which possess varied cinnamic acid residues linked to glycosyl moieties. A natural explanation for the outstanding stability of these acylated anthocyanins is the occurrence of a most efficient intramolecular copigmentation process bringing together the chromophoric part (anthocyanidin) and the cinnamic acid residues in a folded conformation. This has been convincingly demonstrated by the observation of long range NOE effects and of chemical shifts of the cinnamic protons which lie markedly upfield with respect to the analogous methyl cinnamate taken as a reference.[27,30] In the case of diacylated anthocyanins, a sandwich-type structure where the two cinnamic residues stack on both sides of the chromophore has been proposed. We here report on the first quantitative thermodynamic investigation of intramolecular copigmentation based on the comparative study of the hydration reaction for a series of anthocyanins having the same pelargonidin chromophore and which differ in the number and the position of the residues of cinnamic acid derivative (caffeic acid). These anthocyanins are 3-*O*-(2-*O*-β-D-glucopyranosyl-β-D-glucopyranosyl)-5-*O*-β-D-glucopyranosyl pelargonidin (**1**), 3-*O*-(6-*O*-(*trans*-caffeyl)-2-*O*-β-D-glucopyranosyl-β-D-glucopyranosyl)-5-*O*-β-D-glucopyranosyl pelargonidin (**2**), 3-*O*-(2-*O*-(6-*O*-(*trans*-3-*O*-(β-D-glucopyranosyl)caffeyl)-β-D-glucopyranosyl)-β-D-glucopyranosyl)-5-*O*-β-D-glucopyranosyl pelargonidin (**3**), and 3-*O*-(2-*O*-(6-*O*-(*trans*-3-*O*-(β-D-glucopyranosyl)caffeyl)-β-D-glucopyranosyl)-6-*O*-(*trans*-caffeyl)-β-D-glucopyranosyl)-5-*O*-β-D-glucopyranosyl pelargonidin (**4**) (Chart I). They have been isolated from red-purple cultivars of *Pharbitis nil* (morning glory).[22] Their structures have been elucidated by FAB mass spectrometry and ^{1}H NMR including ^{1}H–^{1}H COSY and NOE difference measurements. Preliminary experiments[22] on color stability have shown that **1** is very unstable in neutral solution, whereas **2** and **3** are moderately stable and **4** fairly stable. Thus, the caffeyl residues are effective in opposing the hydration of the pelargonidin chromophore. In this work, we show the marked influence of the caffeyl residues on both the position and the kinetics of the hydration equilibrium of **2**, **3**, and **4** with respect to **1**. These differences are reasonably accounted for by the molecular interaction of the anthocyanin chromophore with one or two of its caffeyl residue(s) and allow for the three

Chart I. Chemical Structures of Pigments **1–4** When in the Flavylium Form AH$^+$ (from Ref 22)



acylated pigments a thermodynamic study of the conformational equilibrium connecting the unprotected open colored forms on the one hand and the protected (hydration-resistant) folded colored forms on the other hand, that is, the intramolecular copigmentation equilibrium. In particular, the distribution of open and folded forms in water will be estimated. Our data concern conformational distributions at equilibrium since the intramolecular copigmentation interaction is an extremely fast process whose rate is not limited by diffusion of the reactants and which probably occurs on the picosecond time scale. Finally, some experiments of intermolecular copigmentation of the pigments **1–4** with chlorogenic acid are reported. Chlorogenic acid (5-caffeylquinic acid), a widespread highly water-soluble natural copigment, is taken here as a reference of external copigment. Its intermolecular association with pigments **2**, **3**, and **4** competes with the intramolecular process, and this will bring additional evidence of the outstanding efficiency of intramolecular copigmentation with respect to intermolecular copigmentation.



Site numbering on anthocyanidin

Chlorogenic acid

Experimental Section

Materials. Pigments **1–4** were isolated from red-purple corolla limbs of *Pharbitis nil* cultivars according to an already published procedure.[22]

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Chlorogenic acid was purchased from Roth. Its purity was checked by UV–vis and ^{1}H NMR spectroscopies.

Absorption Spectra. Spectra were recorded with a Hewlett-Packard diode-array spectrometer fitted with a quartz cell (d = 1 cm) equipped with a stirring magnet. A constant temperature in the cell was obtained by use of a Lauda water-thermostated bath. Temperature was measured with a Comark thermocouple and was kept at 25(±0.1) °C throughout this work.

Kinetic Measurements. One milliliter of an equilibrated solution of anthocyanin in 0.1 M acetic acid (pH about 2.6) is magnetically stirred in the spectrometer cell. The concentrations of pigment are 2×10^{-4} M for **1** and **2**, 10^{-4} M for **3**, and 5×10^{-5} M for **4**. One milliliter of NaOH solution of concentration ranging from 5×10^{-3} to 5×10^{-2} M is quickly added to the cell, and the visible absorbance at a wavelength close to the absorption maximum (usually 510 nm) is immediately recorded every second over 1 min, i.e., until attainment of the hydration equilibrium. The final pH value is carefully measured and ranges from 3.2 to 4.4. The ionic strength is fixed by NaCl and is 0.5 M after mixing. The spectrometer software automatically computes the first-order apparent rate constant of the hydration reaction (k). At least ten (pH, k) pairs are used in the calculations.

Thermodynamic Measurements. (i) Hydration Equilibrium. After the kinetic measurements, the pigment solutions are collected. Some of them are acidified by concentrated HCl (without significant dilution) so that the pH covers a larger domain ranging from 1 to 4.4. The UV–visible spectra are then recorded. The values of the hydration equilibrium constants are gained from measuring the relative hyperchromic shift (at a given wavelength) as a function of pH, the pigment concentration being held constant.

(ii) Intermolecular Copigmentation Equilibria. Pigment and copigment titrated solutions are prepared by dissolving the compounds into a citrate buffer whose pH has been adjusted to the desired value (pH about 3.5 for flavylium ion copigmentation; pH about 5.5 for quinonoidal base copigmentation). A given volume of the pigment solution is mixed with a variable volume of copigment solution and the resulting solution is brought to a constant volume by buffer addition. The values of the copigmentation equilibrium constants are gained from measuring the visible absorbance (at a given wavelength) of equilibrated solutions of pigment at different overall concentrations of copigment, the pH and the pigment concentration being held constant.

Results

Kinetic Measurements. So far, comparing the resistance to fading of a series of anthocyanins, as a function of the acylation pattern, has been carried out in a qualitative way upon dissolving the pigments, under neutral conditions, with consecutive recording of the visible absorbance versus time. However, for a convenient quantitative interpretation of the results, it is better to operate as follows. In a typical experiment, a NaOH solution is quickly added to an equal volume of a fairly acidic equilibrated solution of pigment (in 0.1 M acetic acid) so that the pH of the pigment containing medium is shifted toward a less acidic value (pH-jump). The exponential decay of the visible absorbance (at a given wavelength) recorded at once on the spectrometer essentially reflects the relaxation of the pH-dependent acid-catalyzed hydration equilibrium according to an apparent first-order kinetics. A theoretical treatment (see Appendix A) based on relaxation kinetics leads to eq 1 which expresses the first-order apparent hydration rate constant (k) delivered by the spectrometer software as the reciprocal of the time constant of the exponential

$$k(1 + K_a 10^{pH}) = k_1 + k_2 K_a + k_2 10^{-pH} \qquad (1)$$

decay. For each anthocyanin, k_1, k_2, and K_a represent the hydration rate constant, the rate constant for the reverse process (dehydration), and the acidity constant of the flavylium ion–quinonoidal base pair, respectively. Moreover, the hydration equilibrium constant K_h is expressed as the k_1:k_2 ratio. Thus, replacing k_1 by $k_2 K_h$ in eq 1, we get $k(1 + K_a 10^{pH}) = k_2(K_a + K_h + 10^{-pH})$, which can be rearranged as eq 2. The sum $K_a + K_h$ can be gained from absorbance measurements on equilibrated pigment solutions as a function of pH (see below eq 3). Therefore,

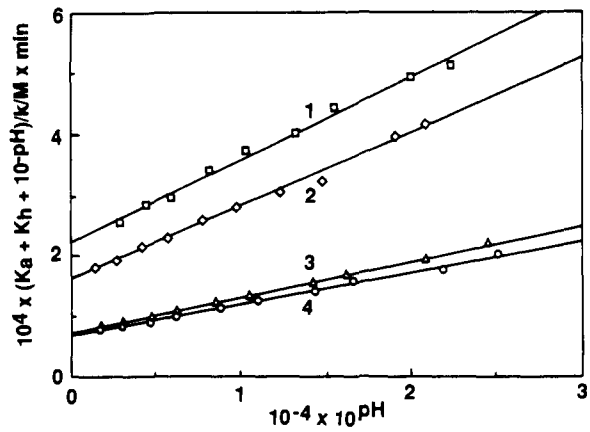

Figure 1. Plots of $(K_a + K_h + 10^{-pH})/k$ versus 10^{pH} for pigments **1**–**4**: T = 25 °C; I = 0.5 M. Concentrations of pigment after pH-jump are 10^{-4} M for **1** and **2**, 5×10^{-5} M for **3**, and 2.5×10^{-5} M for **4**.

Table I. Thermodynamic and Kinetic Parameters for Pigments **1**–**4** at 25 °C and I = 0.5 M[c]

	pigment			
	1	**2**	**3**	**4**
k_1[a]	22.7 (±2.0)	13.4 (±0.9)	12.4 (±1.5)	4.0 (±0.8)
$k_2 \times 10^{-3}$[b]	4.5 (±0.2)	6.3 (±0.2)	14.8 (±0.3)	14.2 (±0.6)
pK_a	4.21 (±0.03)	4.12 (±0.03)	4.05 (±0.02)	4.14 (±0.04)
pK_h	2.30 (±0.03)	2.67 (±0.02)	3.08 (±0.05)	3.55 (±0.07)
K (M^{-1})	76 (±4)	50 (±3)	81 (±5)	22 (±2)
K' (M^{-1})	38 (±3)	30 (±3)	33 (±3)	13 (±2)

[a] Min^{-1}. [b] M^{-1} min^{-1}. [c] For definitions, see text.

plotting $(K_a + K_h + 10^{-pH})/k$ versus 10^{pH} yields a straight line

$$(K_a + K_h + 10^{-pH})/k = 1/k_2 + K_a 10^{pH}/k_2 \qquad (2)$$

with a slope and an intercept equal to K_a/k_2 and $1/k_2$, respectively (Figure 1). From k_2, K_a, and $K_a + K_h$, k_1 and K_h are immediately obtained. k_1 is a convenient reliable parameter for comparing the stability of anthocyanins toward hydration, i.e., fading. The smaller the k_1 value, the more resistant the pigment to fading. As shown in Table I, the presence of one or two caffeyl residue(s) markedly affects the k_1 value, whereas the corresponding variations in the k_2 value seem relatively small. The k_1 values for the monoacylated pigments **2** and **3** are almost identical and about twice as small as for pigment **1**. Pigment **4** turns out to be by far the most resistant to hydration, its k_1 value being about six times as small as the k_1 value for pigment **1**.

Thermodynamic Measurements. (i) Hydration Equilibrium. Recording the visible absorbance of equilibrated anthocyanin solutions at different pH values, the concentration of the pigment and the wavelength being held constant, allows the determination of the hydration thermodynamic constant (K_h) according to eq 3 (see Appendix B). D_0 is the absorbance of a strongly acidic solution (pH < 1) in which the anthocyanin is exclusively under

$$\frac{D_0}{D_0 - D} = \frac{K_h + K_a}{K_h + K_a(1 - r_A)} + \frac{10^{-pH}}{K_h + K_a(1 - r_A)} \qquad (3)$$

the flavylium form. D is the absorbance of an anthocyanin solution at a given slightly acidic pH. r_A stands for the following ratio of molar absorption coefficients: $\epsilon_A/\epsilon_{AH^+}$. Plotting $D_0/(D_0 - D)$ versus 10^{-pH} results in a straight line and the ratio intercept/slope yields $K_h + K_a$ (Figure 2). For pigments **1**, **2**, and **3**, $K_h + K_a$ can be approximated to K_h ($K_a \ll K_h$). Strictly speaking, K_h is an apparent equilibrium constant since the hemiacetal product is in very fast equilibrium with small amounts of (*E*)-

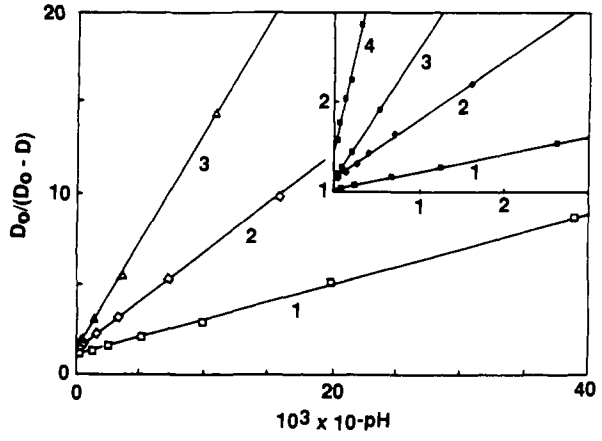

Figure 2. Plots of $D_0/(D_0-D)$ versus 10^{-pH} for pigments 1–4: $T = 25$ °C; $I = 0.5$ M. Concentrations of pigment are 10^{-4} M for 1 and 2, 5×10^{-5} M for 3, and 2.5×10^{-5} M for 4.

chalcone arising from central ring opening.[35,36] It can be reasonably assumed that the difference between our K_h value and the true equilibrium constant for pure hemiacetal formation (K_h^0) is small. Moreover, K_h can be expressed as $K_h^0(1 + K_T)$ where K_T stands for the equilibrium constant of the hemiacetal–(*E*)-chalcone tautomerism. Since K_T is expected to be almost the same for the anthocyanins **1–4**, the ratios of K_h values used in the Discussion to study the intramolecular copigmentation can be readily approximated to the corresponding ratios of K_h^0 values and thus refer to the hydration process, only. Finally, the presence in fully equilibrated anthocyanin solutions of small amounts of the (*Z*)-chalcone arising from very slow *Z*–*E* isomerization of the (*E*)-chalcone can be neglected. Indeed, quick acidification to pH < 1 of a slightly acidic equilibrated solution of pigment results in an instantaneous gain of color through regression of the hydration equilibrium which is fast in a strongly acidic solution. Then, the (*Z*)-chalcone slowly reverting toward the flavylium ion[37,38] is expected to produce a slow additional gain of color which has turned out to be very small, thus indicating that the (*Z*)-chalcone concentration is less than 10% of the total concentration of pigment, whatever the anthocyanin in the series investigated. Therefore, the thermodynamic estimation of K_h (carried out on fully equilibrated solutions) may actually be taken equal to k_1/k_2 in the calculations. The K_h value gives access to the relative amounts of flavylium ion and hemiacetal at equilibrium at a given pH and can be considered a static measure of the resistance of the pigment to hydration. The smaller the K_h value (the larger the pK_h value), the more resistant the pigment to hydration. The resistance of anthocyanins to hydration has been found to increase according to the order **1 < 2 < 3 < 4** (Table I).

(ii) Intermolecular Copigmentation Equilibria. Chlorogenic acid is a natural anthocyanin copigment whose structure is close to that of the (6-caffeyl)glucosyl residues of our acylated pigments, the quinic moiety mimicking the glucosyl moieties. Copigmentation experiments in which chlorogenic acid competes with the (6-caffeyl)glucosyl residues for associating with the anthocyanidin chromophore are expected to yield information about the strength of the intramolecular interaction. The binding constants for copigmentation of pigments **1–4** with chlorogenic acid have been determined from eqs 4 and 5. Equation 4 is valid at pH < pK_a and refers to flavylium ion copigmentation. Equation 5 is valid at pH > pK_a and refers to quinonoidal base copigmentation. Both equations are simplified forms derived from a more general theoretical treatment accounting for pH effect on copigmentation[7]

$$K = \frac{D - D_0}{L_t(rD_0 - aD)} \tag{4}$$

$$K' = \frac{D - D_0}{r'L_tD_0} \tag{5}$$

(see Appendix B). D_0 and D are the visible absorbances of the anthocyanin in the absence and in the presence of the copigment (total concentration L_t), respectively, the wavelength and the pH being fixed. r and r' stand for the following ratios of molar absorption coefficients: $r = \epsilon_{AHL}{}^+/\epsilon_{AH}{}^+$; $r' = \epsilon_{AL}/\epsilon_A$. Finally, a is $1/(1 + K_h10^{pH})$. r can be estimated as the ratio of the visible absorbance of a strongly acidic (pH < 1) anthocyanin solution with an ca. 1000-fold excess of copigment (pure AHL^+) over the absorbance of the corresponding solution without copigment (pure AH^+). Except with **4** for which r is about 1.0, r takes for **1**, **2**, and **3** the usual value of 0.8.[6,7] r' is more difficult to estimate because the hydration process does not allow to place anthocyanins under pure quinonoidal forms. From earlier results on common anthocyanins,[7] we assume r' to be close to 0.9 for the four anthocyanins of our series. For each anthocyanin, the values of K and K' collected in Table I are mean values calculated from series of measures made at different concentrations of copigment. In the case of pigment **4**, remarkably small values for K and K' have been found.

Discussion

Let us first mention that the presence of caffeyl residues in the anthocyanin structure not only confers stability on the color of pigment aqueous solutions but also allows color diversification. Indeed, going from **1** to **4**, the wavelength of absorption maximum in the visible range shifts from 496 to 510 nm for the flavylium chromophore. Bathochromic shifts of such magnitude are frequent in intermolecular copigmentation of common anthocyanins with good copigments in the flavonoid series.[3] We ascribe them to a decrease in the local polarity of the flavylium chromophore upon its hydrophobic association with the copigment. In this work, we essentially focus on the much more unusual color stability of caffeylated anthocyanins with respect to common unacylated anthocyanins. Recently,[9] UV–visible investigations of intermolecular copigmentation have yielded the main thermodynamic parameters (binding constant, enthalpy and entropy changes) of the copigmentation equilibrium of the common malvin ($R_1 = R_2 = OMe$, R = glucosyl in Scheme I) with a series of varied copigments, most of them, like chlorogenic acid for instance, being natural copigments of anthocyanins. These copigments were selected among natural compounds of reasonable water-solubility and also possessing a planar aromatic moiety allowing their stacking onto the planar anthocyanidin. From that study,[9] it could be concluded that copigmentation is an exothermic molecular association with a negative unfavorable entropy change (as already outlined in preliminary investigations[6,39]). Part of this unfavorable ΔS arises from the inevitable reduction in the degrees of freedom of both interacting partners upon association within the molecular complex. Thus, the presence of a covalent link between the anthocyanidin and the copigment (intramolecular copigmentation) is expected to result in a more favorable ΔS since the association will now take place from a structure in which the degrees of freedom are already reduced because of the covalent link. Of course, this covalent link or spacer must orient the copigment so that it can actually interact with the anthocyanidin without bringing additional steric strains which would lower the exothermicity of the association. Most examples of intramolecular

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Chart II. Gentiodelphin (from Ref 30)

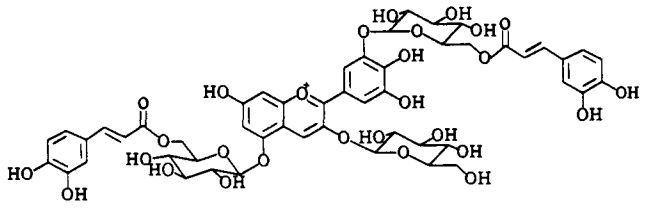
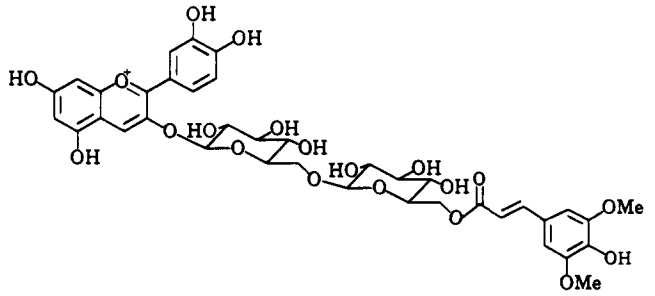


Chart III. Alatanin C (from Ref 40)



copigmentation in nature concern polyacylated anthocyanins with glycosyl moieties as spacers and residues of cinnamic acid derivative as copigments.[2,27,30] However, the position of the cinnamic residue on the glycosylic spacer, the position of the spacer on the anthocyanidin and the length of the spacer (mono- or diglycoside) are certainly determining for a strong copigmentation interaction. For instance, ^{1}H NMR measurements on the diacylated gentiodelphin pigment[30] (Chart II) clearly demonstrate that only the caffeyl residue at C-6 of the glucosyl moiety on the 3′-position of the delphinidin chromophore efficiently takes part in intramolecular copigmentation, whereas the caffeyl residue at C-6 of the glucosyl moiety on the 5-position does not significantly interact with the anthocyanidin nucleus. On the other hand, alatanin C (Chart III), a relatively simple anthocyanin with a sinapic acid residue linked to the cyanidin chromophore through a β-diglucosyl spacer, has been reported to be surprisingly stable for a monoacylated anthocyanin.[40] This large spacer probably allows a good copigmentation interaction resulting in a color stability similar to that achieved in more sophisticated polyacylated anthocyanins upon probable sandwich-type complex formation. Consistent observations have been reported from NOE data on a series of monoacylated cyanidin derivatives with a β-D-glucosyl-β-galactoside spacer, the aromatic acid residues being either cinnamic or benzoic acid derivatives.[41] Returning to our series of anthocyanins, we can see from the k_1 values that none of the two monoacylated anthocyanins **2** and **3** shows a resistance to hydration much larger than that of the unacylated pigment **1**. For **2**, the sophorosyl moiety provides a spacer including four C–O and one C–C bonds between the oxygen atom at the 3-position of the pelargonidin chromophore and the caffeyl ester group. For **3**, the spacer is extended to six C–O and two C–C bonds. By comparison, the diglucosyl spacer in the stable alatanin C includes eight C–O and two C–C bonds. In the case of pigments **2** and **3**, the spacers could be too short to allow a close copigmentation contact and thus an efficient protection of the chromophore against water nucleophilic attack at the 2-position. A decisive improvement in stability is achieved in pigment **4** due to the cooperative effects of the two caffeyl residues which probably stack on both sides of pelargonidin (sandwich-type complex). This is reflected not only in the small values of k_1 and K_h but also in the intermolecular copigmentation binding constants K and K' which have been found about three times as small as the corresponding values for pigment **1**. Interestingly, the values for K and K' do not seem to be significantly affected by the presence of only one caffeyl residue (with respect to **1**), probably because one side of the planar chromophore is still available for interacting with chlorogenic acid. In the case of **4**, the much smaller values for K and K' point to a chromophore less accessible on its two sides, and this is one more argument in favor of sandwich-type complex formation. Previous NMR investigations on stable diacylated anthocyanins[2,27,30] have been recorded on strongly acidic solutions and concern the flavylium ion, only. The long-range NOE effects observed are good evidence of the interaction between the flavylium chromophore and both cinnamic acid residues but can be ascribed to half-folded conformations in extremely fast equilibrium as well as to a genuine sandwich-type conformation. Quantitative UV–visible investigations are expected to clarify that point and also to give some information on the conformational distribution of the quinonoidal bases.

Conformational Equilibria of Acylated Flavylium Ions. The decrease in the k_1 values when the number of caffeyl groups in the anthocyanin structure increases clearly points to the slowing down of the hydration process caused by intramolecular copigmentation, a phenomenon which has already been reported in the case of intermolecular copigmentation.[9] This slowing down is expected to occur essentially through stabilization of the initial state (flavylium ion). Indeed, the flavylium ion is characterized by a large planar chromophore with an electronic delocalization spread over the whole 2-phenyl-1-benzopyrylium nucleus, allowing a strong π-stacking interaction with a caffeyl residue. This outstanding feature largely disappears in the tetrahedral transition state of the hydration reaction and is completely absent in the final state (hemiacetal). Indeed, the hemiacetal, whose chromophore is made up of two unconjugated aromatic rings connected by a nonplanar ring, does not show propensity for π-stacking. For instance, no experimental evidence has been reported so far for the possible taking part of the hemiacetal in formation of intermolecular complexes between anthocyanins and their copigments. In summary, a cinnamic acid residue (at a suitable position in the anthocyanin structure) is expected to interact with the tricyclic system of the pigment strongly within the flavylium form, much more weakly within the hydration transition state and not at all within the hemiacetal form. As a consequence, intramolecular copigmentation must increase the activation free energy of hydration and eventually slightly decrease the activation free energy of the reverse process (hemiacetal dehydration), the influence on the corresponding rate constants k_1 and k_2 being opposite. This is rather well reflected in the values for k_1 and k_2 collected in Table I. In particular, a small but significant speeding up of hemiacetal dehydration has been recorded for pigments **3** and **4** with respect to **1**.

(i) Monoacylated Pigments. Intramolecular copigmentation markedly affects the free energy of the flavylium ion and leaves unchanged the free energy of the hemiacetal. Therefore, the difference in free energy change of the hydration reaction between a monoacylated anthocyanin in which intramolecular copigmentation occurs through one folded conformation (pigment **i** with **i** being equal to **2** or **3** in this work) and the corresponding unacylated anthocyanin (pigment **1**) should provide information about the stabilization occurring in the copigmented conformation of the flavylium ion. For this purpose, we assume that the difference in free energy between hemiacetals **i** and **1** equals the difference in free energy between flavylium ions **i** and **1**, **i** being taken in a hypothetical molecular state consisting in the open conformation, only. Thus, everything happens in the calculations as if hemiacetals **i** and **1**, on the one hand, and flavylium ion **1** and flavylium ion **i** in its open conformation, on the other hand, could be made identical. Now, let us note $\Delta G_h^i = -RT \ln K_h^i$ the hydration free energy change of pigment **i** and $\Delta G_h^1 = -RT \ln K_h^1$ the hydration free energy change of pigment **1**. Then, $\Delta G_i^* = \Delta G_h^1 - \Delta G_h^i = -RT \ln (K_h^1/K_h^i)$ represents the free

(40) Yoshida, K.; Kondo, T.; Goto, T. *Tetrahedron Lett.* **1991**, *32*, 5579–5580.

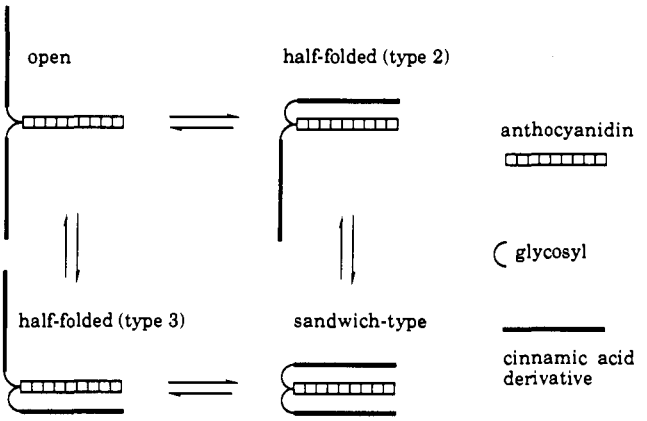
(41) Glässgen, W. E.; Wray, V.; Strack, D.; Metzger, J. W.; Seitz, H. U. *Phytochemistry* **1992**, *31*, 1593–1601.

Table II. Conformational Distribution (in Percentage) for Pigments **2**–**4** at 25 °C and I = 0.5 M[b]

	pigment		
	2	**3**	**4**
K_i*	1.34 (±0.13)	5.03 (±0.80)	10.4 (±4.7)
open	43 (±3)	17 (±3)	6 (±2)
folded (2-type)	57 (±3)		7 (±5)
folded (3-type)		83 (±3)	28 (±15)
sandwich-type			59 (±19)
K_i*′	1.88 (±0.52)	7.71 (±2.03)	10.3 (±12.4)
open	35 (±8)	12 (±3)	*a*
folded (2-type)	65 (±8)		*a*
folded (3-type)		88 (±3)	*a*
sandwich-type			*a*

[a] No value is given because the experimental uncertainty is too great. [b] Upper part: flavylium ion. Lower part: quinonoidal bases. For definitions, see text.

Scheme II. Four Conformations of a Diacylated Anthocyanin in Solution



energy of the virtual equilibrium connecting the open conformation of flavylium ion **i** (flavylium ion **1** in our model) and the mixture of open and folded conformations of flavylium ion **i** in true conformational equilibrium. The equilibrium constant of the virtual equilibrium is thus simply expressed as a ratio of hydration equilibrium constants according to eq 6 where $[AH^+]^f$ and $[AH^+]^0$ stand for the concentrations of the flavylium ion in the folded and open conformations, respectively. From eq 6, the equilibrium constant of the true conformational equilibrium K_i* is immediately gained (eq 7) ($\Delta pK_h^i = pK_h^i - pK_h^1$) and can be defined as the intramolecular copigmentation constant. Using eq 7, the distribution of open and folded conformations has been estimated for flavylium ions **2** and **3** (Table II).

$$([AH^+]^f + [AH^+]^0)/[AH^+]^0 = K_h^1/K_h^i \quad (6)$$

$$K_i^* = [AH^+]^f/[AH^+]^0 = K_h^1/K_h^i - 1 = 10^{\Delta pK_h^i} - 1 \quad (7)$$

(ii) Diacylated Pigments. The situation is more complicated in that case because of the simultaneous presence in solution of four different conformations: a completely open conformation, two half-folded conformations with one of the two cinnamic acid residues stacked onto the anthocyanidin and a completely folded conformation (sandwich-type) (Scheme II). The theoretical treatment applied to **2** and **3** can be easily generalized to **4** with similar assumptions. Thus, flavylium ion **1** is considered a model of the open conformation of flavylium ion **4** (concentration $[AH^+]^0$) and, similarly, flavylium ions **2** and **3** in their folded conformation represent the two half-folded conformations (concentrations $[AH^+]^{f2}$ and $[AH^+]^{f3}$, respectively). To estimate the ratio of half-folded to open conformations, eq 7 still applies and gives $K_2^* = [AH^+]^{f2}/[AH^+]^0 = 10^{\Delta pK_h^2} - 1$ and $K_3^* = [AH^+]^{f3}/[AH^+]^0 = 10^{\Delta pK_h^3} - 1$. Respecting the sandwich-type conformation (concentration $[AH^+]^s$), we consider the virtual equilibrium connecting flavylium ion **4** in its open conformation and the true flavylium ion **4** present in solution as a mixture of four conformations. The corresponding free energy change is approximated to $\Delta G_h^1 - \Delta G_h^4 = -RT \ln (K_h^1/K_h^4)$. The equilibrium constant is then expressed according to eq 8. From eq 8, the ratio of sandwich-type to open conformations (K_4*) is directly obtained (eq 9). Finally, using the K_i* values (i = 2, 3, and 4), the distribution of open, half-folded and sandwich-type conformations for flavylium ion **4** can be estimated (Table II). Clearly, formation of a sandwich-type complex as the predominant conformation is necessary to account for the largely improved resistance of the flavylium chromophore to hydration when going from the monocaffeylated pigments **2** and **3** to the dicaffeylated pigment **4**.

$$([AH^+]^0 + [AH^+]^{f2} + [AH^+]^{f3} + [AH^+]^s)/[AH^+]^0 = K_h^1/K_h^4 \quad (8)$$

$$K_4^* = [AH^+]^s/[AH^+]^0 = 10^{\Delta pK_h^4} - 1 - K_2^* - K_3^* \quad (9)$$

Conformational Equilibria of Acylated Quinonoidal Bases. The main role of a copigment (bound to its pigment or not) is to hinder the hydration reaction which takes place on the flavylium ion and not on the quinonoidal base (in fact, two tautomeric forms) which appears at higher pH values.[12] Therefore, as far as flowers and fruits pigmentation is concerned, interaction between the caffeyl groups and the anthocyanidin is not so crucial to color when the quinonoidal base replaces, as chromophore, the flavylium ion. Nevertheless, copigmentation occurs within the whole acidic pH range and even extents in the alkaline domain.[7] Results in Table I show that, in the series studied, pK_h values always remain smaller than the corresponding pK_a values. In fact, if pK_h became larger than pK_a for a given pigment, the hydration reaction would disappear since deprotonation of the flavylium ion would become simultaneously kinetically and thermodynamically favored as compared to hydration of the same ion. Thus, the pK_h values of natural anthocyanins must range approximately between 2 and 4 for the copigmentation color stabilizing process to remain fully effective in the biological media like flowers epidermal cells, for instance. In the same way that hydration equilibrium constants are used to estimate the conformational distribution of an acylated flavylium ion, thermodynamic acidity constants of flavylium ion–quinonoidal base pairs can be used to estimate the conformational distribution of the corresponding quinonoidal base. Similarly, we assume that the free energy difference between flavylium ion **1** and flavylium ion **i** (**i** = **2**, **3**, and **4**) equals that between quinonoidal base **1** and quinonoidal base **i** when the acylated colored forms **i** are taken in the open form. Thus, we may assimilate in the calculations the three open quinonoidal bases **i** (**i** = **2**, **3**, and **4**) to quinonoidal base **1** and, similarly, the two half-folded quinonoidal bases **4** (2-type and 3-type) to the folded quinonoidal bases **2** and **3**, respectively. For the monoacylated pigments, the proton-transfer equilibrium constant can be expressed as eq 10 in which K_i*′ stands for the ratio of folded to open conformations for quinonoidal base **i** (**i** = **2** and **3**; a_{H^+}, hydronium ion activity). From eq 10, K_i*′ can be easily obtained (eq 11; $\Delta pK_a^i = pK_a^i - pK_a^1$). In the case of the diacylated pigment **4**, eq 10 can be readily generalized

$$K_a^i = a_{H^+}\frac{[A]^0 + [A]^f}{[AH^+]^0 + [AH^+]^f} = K_a^1\frac{1 + K_i^{*\prime}}{1 + K_i^*} \quad (10)$$

$$K_i^{*\prime} = 10^{-\Delta pK_a^i}(1 + K_i^*) - 1 \quad (11)$$

as eq 12 which gives access to the corresponding K_4*′ value (eq 13). Since the pK_a values for pigments **1**–**4** are here reported to

be close (Table I), the K_i^* and $K_i^{*\prime}$ values for pigments **2**, **3**, and **4** must be close too and this points to similar conformational distributions for the flavylium ion and the quinonoidal base in these pigments. For pigments **2** and **3**, this is clearly apparent in the percentage distribution given in Table II. Unfortunately, the very large uncertainty in the $K_4^{*\prime}$ value does not allow to give a reasonable estimate for the conformational distribution of the dicaffeylated quinonoidal base **4**.

$$K_a^4 = a_{H^+}\frac{[A]^0 + [A]^{f2} + [A]^{f3} + [A]^s}{[AH^+]^0 + [AH^+]^{f2} + [AH^+]^{f3} + [AH^+]^s} = K_a^1\frac{1 + K_2^{*\prime} + K_3^{*\prime} + K_4^{*\prime}}{1 + K_2^* + K_3^* + K_4^*} \quad (12)$$

$$K_4^{*\prime} = 10^{-\Delta pK_a^4}(1 + K_2^* + K_3^* + K_4^*) - 1 - K_2^{*\prime} - K_3^{*\prime} \quad (13)$$

Competing Inter- and Intramolecular Copigmentation Processes. The results collected in Table I concerning copigmentation of the four anthocyanins by chlorogenic acid deserve a few comments. First, the values for the binding constants K and K' of the unacylated pigment **1** are quite small in comparison with those of 3,5-diglucosylmalvidin (malvin) deduced from the pH-dependence of the copigmentation magnitude in the malvin–chlorogenic acid pair (at 25 °C, K and K' are ca. 300 and 120 M^{-1}, respectively).[7,9] Indeed, the substituent on the 3-position of the chromophore is a glucosyl group in malvin and a much bulkier sophorosyl group in **1** which probably makes more difficult the π-stacking interaction of the caffeyl group onto the chromophore. Note however that the pK_h value for **1** is similar to that found for more common diglucosyl anthocyanins,[42] thus indicating that the bulkiness of the sophorosyl group in **1** does not significantly protect the pyrylium nucleus from the nucleophilic attack of the small water molecule (hydration). The presence of one caffeyl residue in the anthocyanin structure (pigments **2** and **3**) does not exert a well-defined influence on the binding constants of intermolecular copigmentation. Contradictory effects could be at work in this case. For instance, the caffeyl residue of the anthocyanin could weaken the pigment–copigment intermolecular interaction in opposing the access of the copigment to the chromophore but could as well strengthen it in promoting sandwich-type complex formation, the copigment molecule being, for instance, intercalated between the anthocyanidin nucleus and the caffeyl residue. However, the binding constants for pigment **4**-chlorogenic acid complexation are markedly lower than for the other pigments and this concerns flavylium ion copigmentation as well as quinonoidal base copigmentation. In that case, the steric hindrance due to the caffeyl residues stacked on both sides of the chromophore is probably the dominant factor and in a way shuts off the access of the copigment on both sides on the anthocyanidin. Even a 1000-fold excess in chlorogenic acid with respect to **4** does not produce a copigmentation relative hyperchromic shift larger than 10%. The efficiency of intramolecular copigmentation with respect to intermolecular copigmentation can be illustrated in comparing the propensity of a given acylated anthocyanin for hydration with the propensity of the corresponding unacylated anthocyanin for hydration in the presence of a copigment close in structure to the acyl residue taking part in intramolecular copigmentation. For caffeylated anthocyanins, chlorogenic acid fits this requirement. Thus, we have to compare the K_h value of the acylated anthocyanin with the apparent hydration equilibrium constant of the unacylated anthocyanin in the presence of the copigment. These parameters will be designated as K_h^{intra} and K_h^{inter}, respectively. For the anthocyanins investigated in this work, we have $K_h^{intra} = K_h^i$ (with i = 2, 3, or 4), K_h^{inter} being expressed according to eq 14 in the case of flavylium ion copigmentation (AHL^+ is the complex involving the flavylium ion of pigment **1** and the copigment L; its stability constant is K^1). Because of the very large copigment/pigment ratios used, the free copigment concentration can be taken equal to the total copigment concentration (L_t). The K_h^{intra} to K_h^{inter} ratio (eq 15) can be viewed as a quantitative measure of the efficiency of intramolecular copigmentation relative to intermolecular copigmentation. In particular, it equals 1 when hydration of the anthocyanidin nucleus occurs to the same extent ($K_h^{intra} = K_h^{inter}$) with intramolecular and intermolecular protections by a caffeyl group. This occurs for a critical concentration of copigment L_t^c given by eq 16. Values as high as 0.02, 0.07, and 0.22 M have been calculated for pigments **2**, **3**, and **4**, respectively. Similar considerations apply to quinonoidal base copigmentation.

$$K_h^{inter} = a_{H^+}[B]/([AH^+] + [AHL^+]) = K_h^1/(1 + K^1L_t) \quad (14)$$

$$K_h^{intra}/K_h^{inter} = K_h^i(1 + K^1L_t)/K_h^1 = (1 + K^1L_t)10^{-\Delta pK_h^i} \quad (15)$$

$$L_t^c(i) = (10^{\Delta pK_h^i} - 1)/K^1 \quad (16)$$

The equilibrium constants for the apparent hydration of the quinonoidal base are K'^{intra}_h for the acylated anthocyanins and K'^{inter}_h for the corresponding unacylated anthocyanin in the presence of the copigment. We may write $K'^{intra}_h = K_h^i/K_a^i$ (with i = 2, 3, or 4), K'^{inter}_h being expressed according to eq 17 (AL is the complex involving the quinonoidal base of pigment **1** and the copigment L; its stability constant is K'^1). Finally, eqs 18 and 19 yield the $K'^{intra}_h/K'^{inter}_h$ ratio and the critical concentration of copigment. We have found the apparent hydration of the acylated quinonoidal bases **2**, **3**, and **4** to be as large as that of the unacylated quinonoidal base **1** in the presence of 0.05, 0.20, and 0.52 M chlorogenic acid, respectively. As a reference, the water-solubility

$$K'^{inter}_h = [B]/([A] + [AL]) = K_h^1/(K_a^1(1 + K'^1L_t)) \quad (17)$$

$$K'^{intra}_h/K'^{inter}_h = (1 + K'^1L_t)\,10^{(\Delta pK_a^i - \Delta pK_h^i)} \quad (18)$$

$$L'^c_t(i) = (10^{(\Delta pK_h^i - \Delta pK_a^i)} - 1)/K'^1 \quad (19)$$

of chlorogenic acid is about 0.1 M at 25 °C. In each case, the calculated critical concentrations of copigment are very high. That means that, in order to produce deep stable colors from unacylated anthocyanins, nature has to synthesize large amounts of strong copigments in the vacuolar sap of flowers epidermal cells.[43] For the same result, nature has thus achieved a tremendous economy in copigment by linking one or, much better, two copigment molecules to well-chosen sites of the glycosyl residues.

Appendix

A. Kinetic Measurements. We have to take into account the slow hydration equilibrium and the instantaneous acid–base equilibrium between the flavylium ion and the quinonoidal base. The general rate equation for the relaxation process following a first-order perturbation (pH-jump) of the hydration equilibrium can be written as eq 20

$$d\Delta c_B/dt = k_1\,\Delta c_{AH} - k_2(c_H^*\Delta c_B + c_B^*\Delta c_H) \quad (20)$$

For each species i (H^+, B, AH^+), Δc_i is the time-dependent deviation from c_i^*, the concentration of i in the final equilibrium state (reached after perturbation). Now, in the buffered acetic solution, the final pH value is fixed through the instantaneous

(42) Timberlake, C. F.; Bridle, P. *J. Sci. Food Agric.* **1967**, *18*, 473–478.

(43) Asen, S.; Norris, K. H.; Stewart, R. N. *J. Am. Soc. Hortic. Sci.* **1971**, *96*, 770–773.

acetic acid–acetate ion equilibrium, i.e., just after mixing the pigment and NaOH solutions. Thus, during the relatively slow relaxation process of the hydration equilibrium, the pH can be considered constant. Making $\Delta c_H = 0$ in eq 20, we get eq 21

$$d\Delta c_B/dt = k_1\Delta c_{AH} - k_2 c_H^*\Delta c_B \quad (21)$$

The principle of mass conservation applied to the pigment gives

$$\Delta c_{AH} + \Delta c_B + \Delta c_A = 0 \quad (22)$$

For the instantaneous flavylium ion–quinonoidal base equilibrium, the mass law expresses as eq 23

$$c_H^*\Delta c_A = K_a\Delta c_{AH} \quad (23)$$

Equations 21, 22, and 23 are combined in order to express the apparent first-order rate constant k for B change defined by $d\Delta c_B/dt = -k\Delta c_B$. One gets

$$k = k_2 c_H^* + k_1/(1 + K_a/c_H^*) \quad (24)$$

Equation 24 is equivalent to eq 1 in the text (see Results). Moreover, the visible absorbance D can be written as $D = \epsilon_{AH}dc_{AH} + \epsilon_A dc_A$ where d is the optical pathlength. Thus, the absorbance deviation from the equilibrium value D^* is $\Delta D = D - D^* = \epsilon_{AH}d\Delta c_{AH} + \epsilon_A d\Delta c_A = \epsilon_{AH}d\Delta c_{AH}(1 + r_A K_a/c_H^*)$ with $r_A = \epsilon_A/\epsilon_{AH}$. Using eqs 22 and 23, the following expression for ΔD is derived: $\Delta D = -\epsilon_{AH}d\Delta c_B(1 + r_A K_a/c_H^*)/(1 + K_a/c_H^*)$. ΔD is time-dependent in the same way as Δc_B, i.e., $d\Delta D/dt = -k\Delta D$. Thus, the pigment visible absorbance varies with time according to $-dD/dt = k(D - D^*)$. On integration, we get $D = D^* + (D_0 - D^*)\exp(-kt)$ where D_0 stands for the initial absorbance just after pH-jump. Thus, after pH-jump, the time-dependence of the visible absorbance at a fixed wavelength is a simple exponential law with a time-constant equal to $1/k$.

B. Thermodynamic Measurements. (i) Hydration Equilibrium. The visible absorbance of an anthocyanin solution is $D = \epsilon_{AH}dc_{AH} + \epsilon_A dc_A = \epsilon_{AH}dc_{AH}(1 + r_A K_a/c_H)$ with $r_A = \epsilon_A/\epsilon_{AH}$. The overall concentration of pigment writes as $S_t = c_{AH} + c_B + c_A = c_{AH}(1 + (K_h + K_a)/c_H)$. In a strongly acidic solution where the anthocyanin is only under the flavylium form, D becomes $D_0 = \epsilon_{AH}dS_t$. A combination of those relationships gives $D = D_0(1 + r_A K_a/c_H)/(1 + (K_h + K_a)/c_H)$. This can be rearranged as: $(D_0 - D)/D_0 = (K_h + K_a - r_A K_a)/(K_h + K_a + c_H)$. Taking the reciprocal of these latter equations yields eq 3.

(ii) Copigmentation Equilibria. Recently, a general theoretical treatment accounting for pH effect on copigmentation has been derived.[7] It has led to eq 25 expressing the copigmentation relative hyperchromic shift (at a fixed wavelength) as a function of pH and overall concentration of copigment. The different parameters have already been defined in the text (see Results). Equations 4 and 5 in the text (see Results) are simplified versions of the general law. In a pH range where the quinonoidal base can be

$$(D - D_0)/D_0 = \{(1 + \alpha L_t)/(1 + \beta L_t)\} - 1$$

$$\alpha = (rK10^{-pH} + r' r_A K' K_a)/(10^{-pH} + r_A K_a)$$

$$\beta = (K10^{-pH} + K' K_a)/(10^{-pH} + K_a + K_h) \quad (25)$$

neglected ($pH \ll pK_a$), α becomes rK and β becomes $K/(1 + K_h 10^{pH})$ by making $K_a/10^{-pH} = 0$ and $K' = 0$. Hence, eq 25 becomes eq 4. In a pH range where the flavylium ion can be neglected ($pH \gg pK_a > pK_h$), α becomes $r'K'$ and β becomes 0 by making $10^{-pH}/K_h = 0$, $10^{-pH}/K_a = 0$ and $K' = 0$. Hence, eq 25 becomes eq 5.