Proton Transfer in Anthocyanins and Related Flavylium Salts. Determination of Ground-State Rate Constants with Nanosecond Laser Flash Photolysis

Antonio L. Maçanita,*,†,‡ Paulo F. Moreira, Jr.,§ João C. Lima,†,|| Frank H. Quina,§ Chang Yihwa,§ and Carolina Vautier-Giongo§

Instituto de Tecnologia Química e Biológica, ITQB/UNL, Oeiras, Portugal 2780, Departamento de Química, IST/UTL, Lisboa, Portugal, and Instituto de Química, Universidade de São Paulo, São Paulo, Brasil

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A new method for the determination of proton-transfer rate constants in the ground state of anthocyanins and related flavylium salts is described. The method is based on the well-known pK_a shift of phenols on going from ground to the first excited singlet state coupled to the typically very fast excited state proton transfer and very short lifetimes (picosecond range) occurring in these compounds. Under these conditions, a nanosecond light pulse instantaneously shifts the ground-state equilibrium, after which the ground-state transients can be monitored with nanosecond or microsecond resolution. The method is successfully applied to the determination of the deprotonation rate constants, k_d, of two synthetic flavylium salts and a natural anthocyanin (7-hydroxy-4-methylflavylium chloride (HMF), 4′,7-dihydroxyflavylium chloride (DHF), and malvidin-3-glucoside chloride (oenin), respectively) and the protonation rate constants, k_p, of their conjugate quinonoidal bases in the ground state. For all three flavylium cations, the protonation of the ground state base form is essentially diffusion-controlled, and the deprotonation occurs in the submicrosecond range. Our directly determined rate constants are 2 orders of magnitude greater than previous values estimated for oenin by T jump. The flash photolysis approach utilized in the present work seems to be the only technique available for measurement of the kinetics of proton transfer in anthocyanins. In addition, our results show clear laser-induced perturbation of the ground-state protonation of oenin, providing the first direct evidence for excited-state proton transfer as a significant energy dissipation process in natural anthocyanins.

Introduction

Anthocyanins are interesting compounds for several reasons, ranging from the beautiful red or blue colors they impart to most flowers and fruits[1] and their potential value as food dyes[2] to the extraordinary richness of their chemistry. This includes complex multiequilibria in the ground state (coupled acid−base, hydration, tautomerization, and isomerization equilibria)[3−7] and unusual properties in the excited state, such as ultrafast proton transfer[8] and photochromism.[9−12]

The chemical versatility of anthocyanins and their parent synthetic flavylium salts is such that combinations of light and pH can be used to induce chemical changes that mimic logical devices.[13] Obviously, proton-transfer plays a central role in all of this ground and excited-state chemistry of anthocyanins.

The understanding of the above phenomena is presently far from complete in many cases, which is not surprising given the near absence of kinetic data for the processes involved, particularly those involving proton transfer. Although values of deprotonation equilibrium constants are available for several compounds of the anthocyanin family (synthetic flavylium salts and natural anthocyanins),[14−19] values of rate constants are scarce.[3,4,20] A plausible reason is the fact that these rates fall in experimentally "difficult" time domains, i.e., in the nano- to microsecond range in the ground state and the femto- to picosecond range in the singlet excited state.

Ground-state deprotonation rate constants (k_d) of the flavylium cation forms of malvidin 3-glucoside (oenin) and malvidin 3,5-diglucoside (malvin) have been estimated from temperature jump (from 4 to 6.5 °C) experiments. The k_d values found for oenin and malvin, 4.7 (± 0.4) × 10^4 s^{-1} (at 25 °C, $I = 0.2$ M)[3] and 1.8 (± 0.1) × 10^4 s^{-1} (at 6.5 °C, $I = 0.2$ M),[4] respectively, suggested that the back protonation rate was about 2 orders of magnitude slower than diffusion control.

Recently, we investigated the dynamics of the acid−base equilibrium of 7-hydroxy-4-methylflavylium chloride (HMF) in the excited singlet state by picosecond time-resolved fluorescence spectroscopy.[8] To our knowledge, the experimentally determined value of the deprotonation rate constant ($k_d^* = 1.5 \times 10^{11}$ s^{-1}) of the excited singlet state of HMF is the fastest rate measured for an intermolecular deprotonation to water. The rate constant for protonation of the excited singlet state of its conjugate base ($k_p^* = 2.3 \times 10^{10}$ L mol^{-1} s^{-1}) is also large and indicative of diffusion control. These data imply that deprotonation of AH^+ in the excited state (6 ps) and decay of the resultant excited quinonoidal base to the ground state (132 ps)[8] should both be much faster than reprotonation of the quinonoidal base in the ground state (ca. 40 ns at pH = 3, assuming diffusion control). Consequently, excitation should transiently perturb the ground-state protonation equilibrium, implying that, by nanosecond laser flash photolysis, it should be possible to measure the rates of proton transfer *in the ground state* as the system relaxes back to equilibrium. In this paper, we utilize this approach to determine the dynamics of the ground-state protonation equilibria of three representative cases: the synthetic salts HMF and 4′,7-dihydroxyflavylium

* To whom correspondence should be addressed.
† Instituto de Tecnologia Química e Biológica.
‡ Departamento de Química, IST/UTL.
§ Universidade de São Paulo.
|| Present address: Centro de Quìmica Fina e Biológica, Dep. Química, FCT/UNL, Portugal.

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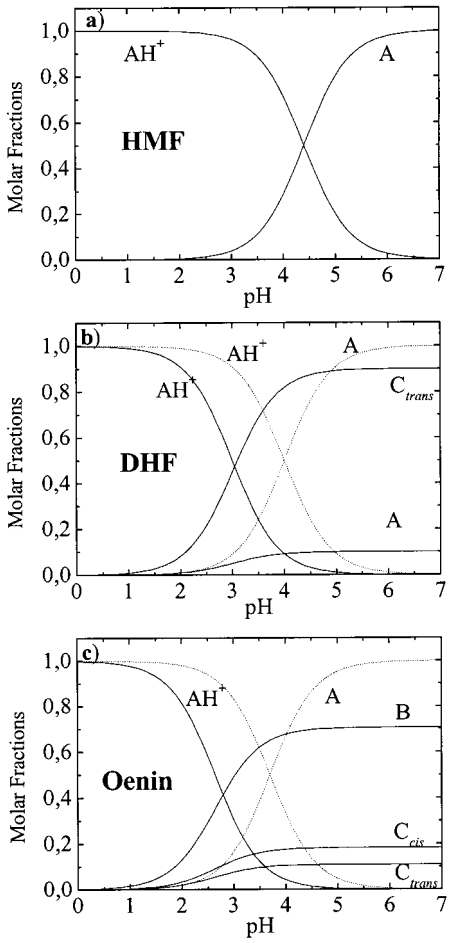


Figure 1. (a) pH dependence of the mole fractions of the flavylium cation (AH^+) and the quinonoidal base (A) of HMF; (b) pH dependence of the mole fractions of the flavylium cation (AH^+), the quinonoidal base (A), and the *trans*-chalcone (C_{trans}) forms of DHF; (c) pH dependence of the mole fractions of the flavylium cation (AH^+), the hemiacetal (A), and the *cis*- (C_{cis}) and *trans*-chalcone forms (C_{trans}) of Oenin (Malvidin 3-glucoside). The dashed lines represent the mole fractions measured immediately after pH jumps, and the continuous lines represent the mole fractions in equilibrated solutions, at 20 °C.

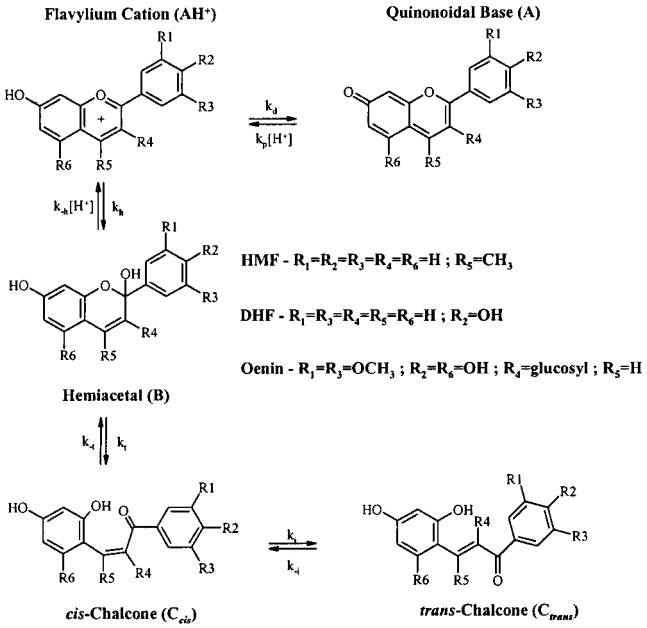
chloride (DHF) and the natural anthocyanin malvidin-3-glucoside chloride (Oenin).

HMF exhibits the simplest possible ground state chemistry of flavylium salts. In moderately acidic solutions, only the flavylium cation, AH^+, and the quinonoidal base, A, are detected. The pH dependence of the mole fractions of AH^+ and A is shown in Figure 1a.

DHF is representative of compounds that exhibit simplified anthocyanin multiequilibria. In addition to the prototropic equilibrium, coupled hydration, tautomerization and isomerization equilibria lead to formation of the hemiacetal, B, and the *cis*- and *trans*-chalcone forms, C_{cis} and C_{trans}, respectively (Scheme 1).[18] However, in the case of DHF, B and C_{cis} are never detected, with C_{trans} being the predominant form at pH > 4.[18] Thus, the system simplifies to the presence of only three forms: AH^+, A, and C_{trans} (Figure 1b).

Oenin exhibits the complex multiequilibria typical of most natural anthocyanins (Scheme 1). The base form, A, is only detectable under special conditions (see the Results section) and is absent in equilibrated solutions. The hemiacetal, B, is the major form at moderately acidic pH (Figure 1c), with a chalcone ($C_{cis} + C_{trans}$):B molar ratio of about 1:2 (Figure 1c).[7] Oenin is also representative of the natural anthocyanins in the sense that

SCHEME 1



both the acid and base forms fluoresce only very weakly, making it difficult or impossible to demonstrate the occurrence of excited-state proton transfer as a significant process on the basis of steady-state or time-resolved fluorescence measurements.

Experimental Section

Compounds. HMF was synthesized by condensation of resorcinol (99%, Aldrich) and 1-benzoylacetone (98%, Aldrich) in glacial acetic acid under a constant flux of gaseous HCl. After 2 h, the flavylium cation began to precipitate. The precipitate was recrystallized from ethanol containing 1% HCl. DMF was prepared by condensation of 2,4-dihydroxybenzaldehyde (98%, Aldrich) and 4-hydroxyacetophenone (98%, Aldrich) in formic acid under a constant flux of gaseous HCl. The flavylium chloride was also obtained as a precipitate, which was recrystallized from acidified ethanol (1% HCl). Oenin chloride (HPLC) was purchased from Extrasynthése and used without further purification. Water was twice distilled and deionized (Elgastat UHQ PS).

Samples. The concentration of the flavylium chlorides was ca. 10^{-5} M. The pH was adjusted with 24 mM phosphate buffers and measured at 20 °C with a Crison micropH 2002. UV−vis absorption spectra were recorded on a Beckman DU-70 spectrophotometer.

pH Jumps. pH jumps from pH = 1 to pH values between 1 and 5.9 were achieved, as before,[18] by mixing a solution of DHF or Oenin in 0.1 M HCl with an equal volume of buffer solution (0.024 M phosphate buffer) of pH close to the desired final pH to which 0.1 M NaOH had been added. The actual final pH was measured at the end of the experiment. The mixing time was around 3 s and the spectra were run in 5 s. The decay of the absorption of the base after pH jumps is slow enough to allow reliable determination of the absorbance of A at time zero and, hence, the extinction coefficients of the base (14 000 and 16 600 L mol^{-1} cm^{-1} for DHF and oenin, respectively, at the maximum in the visible). The acid−base equilibrium is much faster than the remaining equilibria, so that the spectra recorded at time zero, after pH jumps to values of pH between 1 and 5.9, consist of the absorption of AH^+ and A in equilibrium. These species are in a preequilibrium state relative to the other

forms and decay together with time. The relative concentrations of both forms can thus be obtained at any time after the pH jump, once the extinction coefficient of both species is known.

Laser Flash Photolysis. The laser flash photolysis experiments were carried out with an Edinburgh Analytical Instruments LP900 laser flash photolysis system equipped with a Surelite I-10 Nd:YAG laser for excitation and a 450 W high pressure xenon lamp for monitoring transient absorption. Aliquots (3.00 mL) of solutions of the flavylium salt of interest (in 0.025 M phosphate buffer of the desired pH, with an absorbance of approximately 0.5 cm^{-1} at the laser excitation wavelength), contained in standard 1 cm path length quartz fluorescence cuvettes, were excited with the third harmonic (355 nm) or the second harmonic (532 nm) of the Nd:YAG laser. Solutions were stirred between each laser shot, and 10 laser shots were average to obtain the transient absorption decays. Solutions were monitored for laser-induced decomposition by conventional UV−vis absorption spectroscopy (Hewlett-Packard 8452A diode array spectrometer) and replaced by fresh solution at the first signs of decomposition. The standard exponential decay routines of the Edinburgh Analytical Instruments LP900 system software were used to analyze the decays of the transient and obtain the lifetimes of the excited species. Lifetimes shorter than 30 ns were deconvoluted using the pulse shape of the laser (obtained by monitoring the Raman scattering peak of water in the fluorescence mode of the LP900, i.e., monitoring beam shutter closed). Lifetimes longer than 30 ns were obtained from direct fits of the decay (from the maximum of the laser pulse on) without deconvolution. In all cases, a single exponential adequately described the decay. Transient absorption spectra were determined point-by-point and, in all cases, the lifetime of the transient showed no significant variation with wavelength.

Molecular Orbital Calculations. Atomic charge densities were calculated using the Hyperchem software from Hypercube, Inc. (Professional version). The Austin method (AM1) was used both for molecular geometry optimization and charge density calculations in the ground and in the excited state.

Results

HMF. Figure 2a shows the absorption spectra of HMF in aqueous solution at a series of pH values. At pH = 1, only AH^+ is present (λ_{max}= 416.5 nm) and at pH = 6, the equilibrium is completely shifted toward the base form, A (λ_{max}= 464 nm). The pK_a is equal to 4.45.[8]

In Figure 2b, the transient absorption spectrum of a solution of HMF at pH = 4.0, generated by excitation at 355 nm with a 5 ns laser pulse, is presented. Besides the depletion of AH^+ at λ_{max}= 416 nm, a new absorption band peaking at ca. 465 nm is clearly observed. The time evolution of the two transients (recovery at 416 nm and decay at 464 nm) and the statistical data obtained from single-exponential analysis are presented in Figure 3. The decay of the absorption at 464 nm is single-exponential and its lifetime, τ, is equal to the recover time of AH^+. Decay times, measured at several concentrations of H^+, are given in Table 1. Note the increase, from 3.8 to 333 ns, with pH. On the basis of the above observations, the new band is assigned to the absorption of the base form, A, which decays due to protonation back to the flavylium cation.

DHF. Figure 4a shows the absorption spectrum of DHF at pH = 1.0 (absorption of AH^+, λ_{max}= 456 nm) and absorption spectra, taken immediately after a pH jump from pH = 1 to 5.9 and after 3 h, when the hydration and tautomeric equilibria have been established at this new pH. At time zero (t < 10 s), only the quinonoidal base, A, is formed (λ_{max}= 495 nm). This

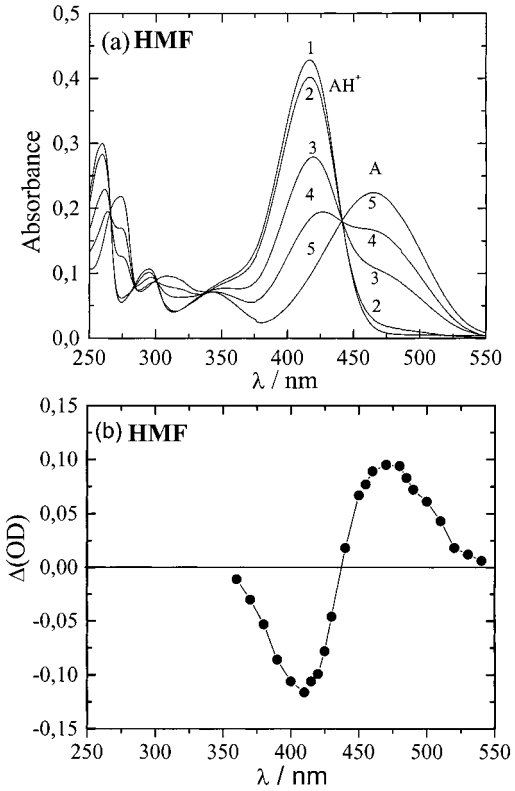


Figure 2. (a) Absorption spectra of HMF ($\sim 1 \times 10^{-5}$ M) in aqueous solution at several pH values: (1) pH = 1.3, (2) 3, (3) 4.4, (4) 4.9, and (5) 6.9. At pH = 1.3, the absorption spectrum is that of the cationic form, AH^+ (λ_{max}= 416.5 nm), and at pH = 6, it is that of the base form, A (λ_{max}= 464 nm). (b) Transient absorption spectra of HMF in aqueous solution at pH = 4.0, measured at time zero after excitation with a 5 ns laser pulse (355 nm). Note the depletion of AH^+ at λ_{max}= 415 nm and the appearance of A at λ_{max}= 465 nm.

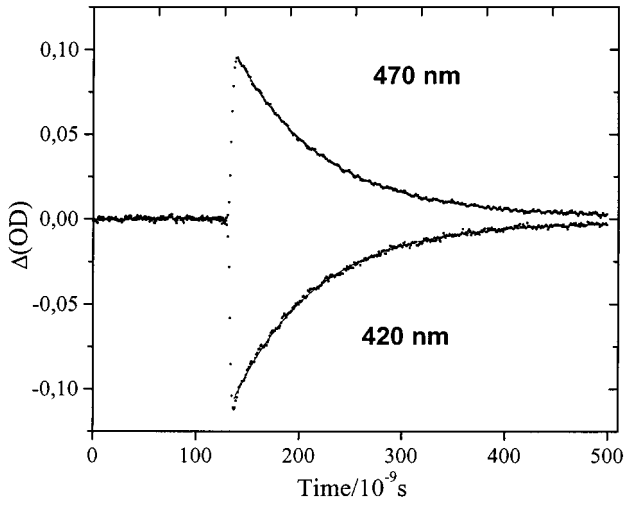


Figure 3. Decay of the base form, A, at 470 nm and recovery of the acid form, AH^+, of HMF at 420 nm, at pH = 4.0, and their respective analyses in terms of single-exponential functions (τ = 169 ns).

is followed by the slow conversion (k_{obs}=3 × 10^{-4} s^{-1} at pH = 5.9) of A into the *trans*-chalcone, C_{trans} (λ_{max}= 370 nm), by way of the flavylium cation.[18] The dashed lines in Figure 1b represent the mole fractions of AH^+ and A measured immediately after pH jumps (pK_a = 4.0) and the full lines represent the mole fractions of AH^+, A, and C_{trans} in equilibrated solutions.[18]

Aqueous solutions of DHF were subjected to laser flash photolysis immediately after preparation, i.e., under conditions

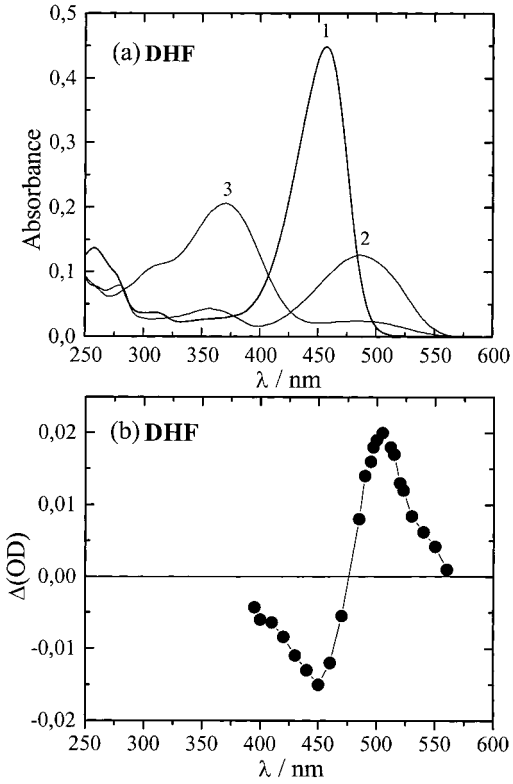


Figure 4. (a) Absorption spectrum of DHF at (1) pH = 1.0, (2) after a pH jump from pH = 1 to 5.9, and (3) at pH = 5.9 after equilibration. (b) Transient absorption spectrum of DHF in aqueous solution at pH = 3.3.

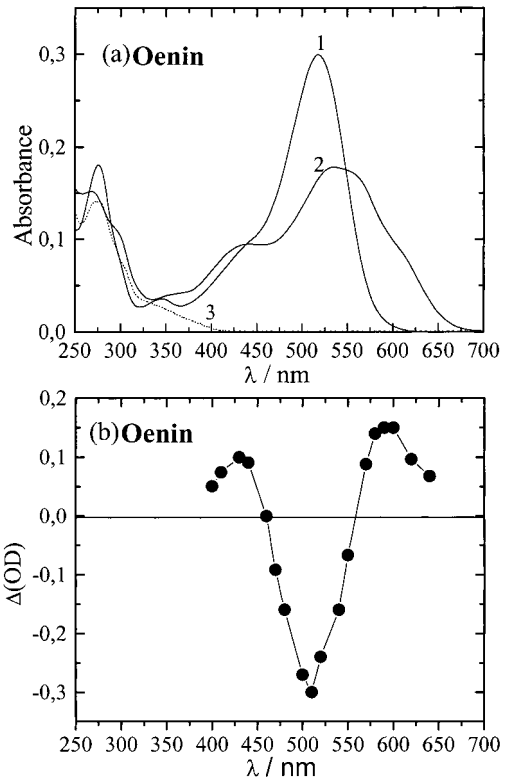


Figure 5. (a) Absorption spectrum of Oenin chloride (1) at pH = 1.0, (2) after a pH jump from pH = 1 to 5.9, and (3) at pH = 5.9 after equilibration. (b) Transient absorption spectrum of Oenin chloride, in aqueous solution, at pH = 3.0.

TABLE 1: Transient Lifetimes, τ (±10%), of the Quinonoidal Base of 7-Hydroxy-4-methylflavylium Chloride, Generated by a 5 ns Pulse (355 nm) from a Nd:YAG Laser in Aqueous Solutions as a Function of pH

HMF		DHF		Oenin	
pH	τ/ns	pH	τ/ns	pH	τ/ns
2.17	3.8	2.48	9.5	2.56	13.3
2.27	5.4	2.53	11.4	2.67	17.1
2.44	8.1	2.60	11.8	2.71	18.2
2.47	9.2	2.69	15.3	2.75	18.6
2.56	10.5	2.76	18.4	2.82	24.1
2.71	14.8	2.82	18.7	2.86	28.2
2.75	15	2.97	29.6	2.95	28.9
3.03	26.1	3.20	46	3.03	35
3.27	45.9	3.40	59	3.10	48
3.29	46.5	3.42	65	3.24	52
3.68	103	3.64	101	3.36	51
3.94	161	3.77	136	3.48	63
4.27	232	4.12	217	3.48	79
4.88	333	5.33	283		

where only AH^+ and A are present (Figure 4b). As seen with HMF, the transient absorption spectra of DHF show only the depletion of AH^+ at 456 nm and the absorption of A at 495 nm. The recovery of AH^+ and the decay of A are both single exponentials with essentially identical lifetimes.

The same solutions were allowed to equilibrate and were subjected to flash photolysis again. The results (spectrum and decays) were identical to those obtained immediately after preparation. This means that the presence of C_{trans} and other equilibrium species does not interfere with the acid−base kinetics.

Oenin. Absorption spectra of Oenin in aqueous solution at pH = 1 and immediately after a pH jump to pH = 5.9 are shown in Figure 5a, together with the spectrum obtained after equilibrium is reached at the latter pH. At pH = 1, the spectrum is the absorption of the acidic form, AH^+ (λ_{max}= 520 nm), as observed with HMF and DHF. The spectrum at pH = 5.9 and t = 0, measured immediately after a pH jump from pH = 1 to 5.9, is assigned to the absorption of A. The absorption of A (λ_{max}= 540 nm) decreases with time (k_{obs}=1.7 × 10^{-3} s^{-1} at pH = 5.9) until it disappears and is replaced by the absorptions that are due to the hemiacetal (λ_{max} = 275 nm) and chalcone forms (λ_{max} = 330 nm). The dashed lines in Figure 1c represent the mole fractions of AH^+ and A measured immediately after pH jumps, giving a pK_a value of 3.7, and the full lines represent the mole fractions of AH^+, B, C_{cis}, and C_{trans} in equilibrated solutions.[7] The transient absorption spectrum of Oenin, measured just after the laser pulse, shows the depletion of the acid form at λ_{max} = 520 nm and two absorption bands at λ = 570 and 440 nm (Figure 5b). These bands are assigned to the absorption of A.

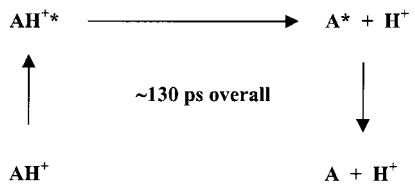
As observed with HMF and DHF, both recovery (520 nm) and decay (570 nm) can be fit with single exponentials with the same lifetime, independent of whether the experiments are carried out immediately after the pH jumps or after equilibration.

Discussion

Kinetic Analysis. *HMF*. The experiments described in the previous paragraphs are interpreted as follows.

The value of the pK_a of HMF in the excited state (pK_a* = −0.81) is 5 pH units lower than its ground-state value (pK_a = 4.40).[8] Consequently, excitation of aqueous solutions of HMF at pH values within the range 2 < pH < 4, where AH^+ is the predominant species present, leads to the rapid formation of A* (in about 6 ps). The excited base then decays with a lifetime of 130 ps to its ground state, A. Thus, the *ground state* acid−base system is, for all practical purposes, instantaneously (within

the 5 ns pulse) shifted out of equilibrium (perturbed toward formation of the base):



Thus, within the laser pulse duration, the four species system is reduced to a simple two species system: the ground-state acidic, AH^+, and base forms, A, of HMF, out of equilibrium.

The time evolution of concentrations after the laser pulse obeys eq 1:

$$\frac{d}{dt}\begin{bmatrix}[AH^+]\\ [A]\end{bmatrix} = \begin{bmatrix}-k_d & k_p[H^+]\\ k_d & -k_p[H^+]\end{bmatrix} \times \begin{bmatrix}[AH^+]\\ [A]\end{bmatrix} \quad (1)$$

with the following boundary conditions at $t = 0$:

$$[AH^+](0) = [AH^+]_{eq} - I_{ef} \quad (2)$$

$$[A](0) = (I_{ef} - [A]_{eq}) \quad (3)$$

In eqs 2 and 3, $[AH^+]_{eq}$ and $[A]_{eq}$ are the concentrations of the acid and base forms at equilibrium and I_{ef} is the ground-state concentration of A generated by the laser pulse. The solutions of eq 1 are eqs 4 and 5, which predict single-exponential recovery and decay, with equal lifetimes of the transients corresponding to AH^+ and A, as experimentally observed:

$$\Delta[AH^+](t) = [AH^+](t) - [AH^+]_{eq} = -(I_{ef} - K_a[AH^+]_{eq})e^{-(k_d+k_p[H^+])t} \quad (4)$$

$$\Delta[A](t) = [A](t) - [A]_{eq} = (I_{ef} - [A]_{eq})e^{-(k_d+k_p[H^+])t} \quad (5)$$

The observed rate constant, k_{obs}, equal to the reciprocal of the transient lifetime, τ, is predicted to be a linear function of the hydronium ion concentration $[H^+]$ (eq 6):

$$k_{obs} = k_d + k_p[H^+] \quad (6)$$

Indeed, as shown in Figure 6a, the plot of k_{obs} as a function of $[H^+]$ is linear over 2 orders of magnitude of τ (Table 1). From the slope of this plot, the value of $k_p = (3.56 \pm 0.09) \times 10^{10}$ L mol^{-1} s^{-1} can be obtained with acceptable accuracy. Unfortunately, the accuracy of the k_d value obtained from the intercept, $k_d = (1.1 \pm 2.4) \times 10^6$ s^{-1}, is very low. This is a consequence of the fact that the experimental observable (reciprocal of the transient lifetime) is the sum of $k_d + k_p[H^+]$ and $k_p[H^+]$ cannot be made much smaller than ca. 10^6 s^{-1} (at pH $\approx$ pK_a = 4.4 with a diffusion-controlled k_p), which in turn represents the lower limit of k_d that can be directly obtained from the intercept. However, from the combination of k_p and $K_a = 3.98 \times 10^{-5}$, a more accurate value of $k_d = 1.4 \times 10^6$ s^{-1} can be obtained.

DHF. Excited-state proton transfer with DHF is similar to HMF and other flavylium salts: fast deprotonation of $(AH^+)^*$ (12 ps) and fast decay of A* (270 ps).[21] This explains the observed formation of A during the light pulse. However, unlike HMF, which is resistant to hydration, the flavylium cation form

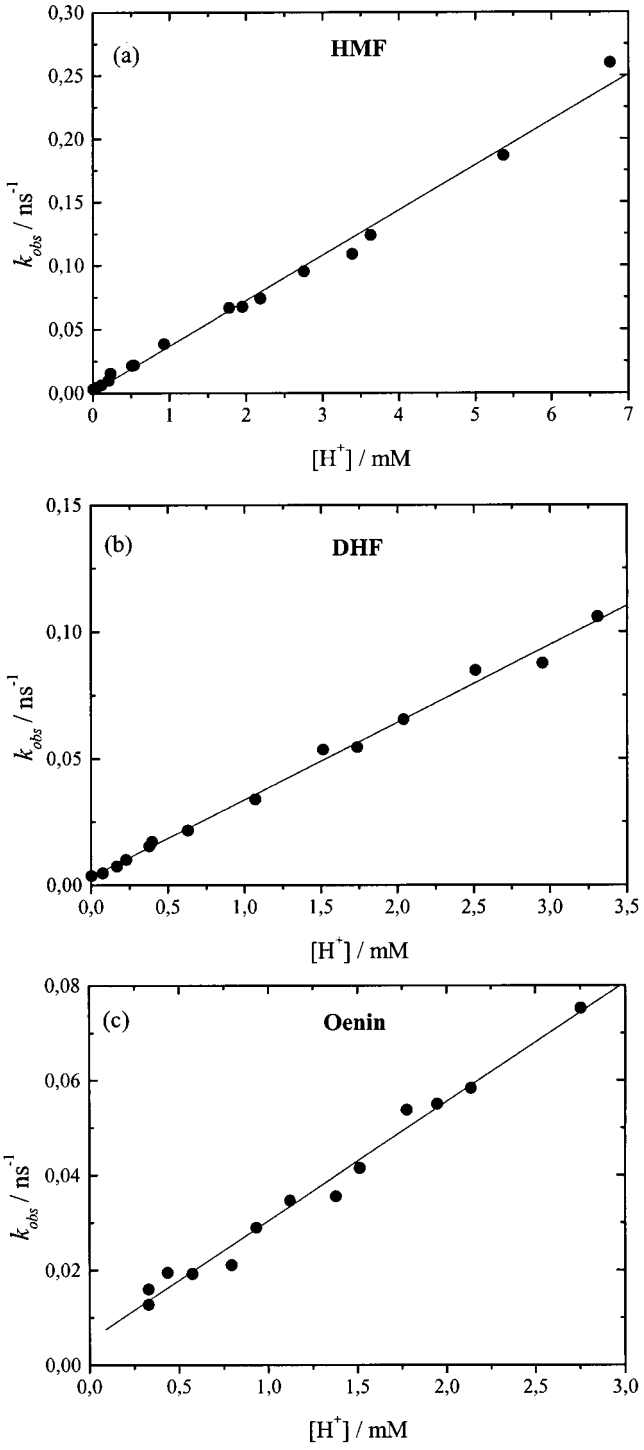


Figure 6. Plot of the reciprocal decay times (k_{obs}) as a function of $[H^+]$ for the base forms of (a) HMF, (b) DMF, and (c) Oenin.

of DMF readily undergoes hydration at higher pHs, requiring that all of the equilibria of Scheme 1 be considered. The fact that B and C_{cis} are never detected allows us to make considerable simplifications to the kinetic scheme; namely, for DHF, Scheme 1 reduces to eq 7:[18]

$$A \underset{k_{a1}}{\overset{k_{-a1}[H^+]}{\rightleftharpoons}} AH^+ \underset{k_{-1}[H^+]}{\overset{k_1}{\rightleftharpoons}} C_{trans} \quad (7)$$

where k_d and k_p are the deprotonation and back-protonation rate constants and k_1 and k_{-1} are related to the rate constants in

Scheme 1 by eqs 8 and 9.[18]

$$k_1 = \frac{k_h k_t k_i}{(k_t + k_{-h}[H^+])(k_i + k_{-t}) - k_t k_{-t}} = \frac{2.9 \times 10^{-7}}{2.7 \times 10^{-5} + [H^+]} s^{-1} \quad (8)$$

$$k_{-1} = \frac{k_{-h} k_{-t} k_{-i}}{(k_t + k_{-h}[H^+])(k_i + k_{-t}) - k_t k_{-t}} = \frac{3.3 \times 10^{-4}}{2.7 \times 10^{-5} + [H^+]} s^{-1} \quad (9)$$

The time evolution of the concentrations of AH^+, A, and C_{trans} can be straightforwardly derived from the appropriate differential eq 10:

$$\frac{d}{dt}\begin{bmatrix}[A]\\ [AH^+]\\ [C_{trans}]\end{bmatrix} = \begin{bmatrix}-k_p[H^+] & k_d & 0\\ k_p[H^+] & -(k_1 + k_d) & k_{-1}[H^+]\\ 0 & k_1 & -k_{-1}[H^+]\end{bmatrix} \times \begin{bmatrix}[A]\\ [AH^+]\\ [C_{trans}]\end{bmatrix} \quad (10)$$

The solutions of eq 10 are double exponential functions

$$[A] = [A]_{eq} + a_2 e^{-\lambda_2 t} + a_3 e^{-\lambda_3 t} \quad (11)$$

$$[AH^+] = [AH^+]_{eq} + b_2 e^{-\lambda_2 t} + b_3 e^{-\lambda_3 t} \quad (12)$$

$$[C] = [C]_{eq} + c_2 e^{-\lambda_2 t} + c_3 e^{-\lambda_3 t} \quad (13)$$

where the preexponential factors (a_i, b_i, and c_i) are relatively complex functions of the initial concentrations, rate constants, and pH. The decay constants λ_3 and λ_2 are given by[18]

$$\lambda_{3,2} = \frac{S \pm \sqrt{D^2 + 4k_d k_1}}{2} \quad (14)$$

with

$$S = k_d + k_p[H^+] + k_1 + k_{-1}[H^+] \quad (15)$$

and

$$D = k_d + k_p[H^+] - k_1 - k_{-1}[H^+] \quad (16)$$

The decay constants λ_2 and λ_3 are essentially determined by the slow hydration−tautomerization−isomerization process and by the fast acid−base equilibrium, respectively. Values of λ_2, measured as a function of pH in a previous work, range between 10^{-4} and 10^{-2} s^{-1};[18] that is, they are 10 orders of magnitude smaller than λ_3 (10^6−10^8 s^{-1}). Thus, the AH^+/A equilibrium is kinetically decoupled from the other equilibria, because of the very different time ranges involved (nanoseconds to microseconds for the AH^+/A equilibrium and seconds to hours for the AH^+/C_{trans} equilibrium). This explains the observation of first order kinetics in all experiments and the lack of dependence of the lifetimes on the presence (equilibrated solutions) or absence (immediately after pH jump) of the other species such as the hemiacetal or chalcone. Furthermore, k_{obs}, given by the reciprocal of the decay time λ_3, is still equal to $k_d + k_p[H^+]$, because $\lambda_3 \approx \lambda_3 + \lambda_2 = S$ and both k_1 and $k_{-1}[H^+]$ are much smaller than k_d and $k_p[H^+]$ (see eqs 8, 9, and 15).

The plot of k_{obs} for the decay of the base form of DHF as a function of the hydronium concentration, shown in Figure 6b, provides an accurate value of $k_p = (3.06 \pm 0.06) \times 10^{10}$ L mol^{-1} s^{-1}. Although the accuracy of the k_d value obtained from the intercept, $k_d = (3.13 \pm 1.10) \times 10^6$ s^{-1}, is still relatively low, it is much better than with HMF and in much closer agreement with the value of $k_d = 3.1 \times 10^6$ s^{-1} calculated from the values of k_p and $K_a = 1.0 \times 10^{-4}$.

Oenin. In the case of Oenin, direct evidence for excited-state deprotonation and determination of the corresponding rate constants is very difficult to obtain because $(AH^+)^*$ is only very weakly fluorescent and there is no detectable fluorescence from A*. Nevertheless, in solvents such as methanol−water mixtures, where deprotonation rates are expected to decrease with respect to water, fluorescence of $(AH^+)^*$ is observed and decay times shorter than 20 ps are obtained.[21] Our interpretation is that the absence of fluorescence in water is due to efficient deprotonation of $(AH^+)^*$. This interpretation is substantiated by the present laser flash experiments with Oenin, which clearly show the transient production of the ground-state base, A.

As with DHF, the observed single-exponential decay of the ground-state base form of Oenin to the ground-state AH^+ is due to the large difference in magnitude between the proton transfer and the hydration rates. The value of the hydration rate constant of Oenin ($k_h = 8.5 \times 10^{-2}$ s^{-1})[3] is 8 orders of magnitude smaller than k_d (see below). Under these conditions, the observed rate constant is also given by eq 6 and is a linear function of $[H^+]$, as shown in Figure 6c. The value of $k_p = (2.51 \pm 0.1) \times 10^{10}$ L mol^{-1} s^{-1} is slightly lower than for HMF and DHF, but $k_d = (5.3 \pm 1.4) \times 10^6$ s^{-1} is clearly larger and its error decreases correspondingly. From k_p and the value of the acidity constant of Oenin, $K_a = 2.0 \times 10^{-4}$, a value of $k_d = 5.0 \times 10^6$ s^{-1} is calculated.

Table 2 summarizes the kinetic data for ground and excited-state proton transfer of HMF, DHF, and Oenin together with calculated charge densities on the hydroxyl oxygen atom at position 7.

Comparison of Ground and Excited-State Proton-Transfer Rates. The large pK_a shift of HMF and DHF upon going from the ground state to the first excited singlet state might, a priori, be due to changes of both the deprotonation and the protonation rate constants. The present results show that the pK_a shift is primarily due to the ca. 5 order of magnitude increase of the deprotonation rate constant in the excited state with respect to the ground state. A substantial decrease in the charge density on the hydroxyl oxygen at position 7 upon electronic excitation is compatible with this observation, as indicated by the calculations in Table 2. Thus, in the excited state, the O−H bond is weakened because of a decrease in the ionic contribution to the bonding, and AH^+ becomes a stronger acid.

The protonation rate constants are in the diffusion-controlled range in all cases. However, for both HMF and DHF, there is a clear decrease of k_p by a factor of ca. 1.5 upon electronic excitation. The experimental accuracy in both the ground and excited-state experiments is much better than this difference. This decrease in k_p is also consistent with the decrease in the charge density of the oxygen at position 7 upon excitation. Thus, the ground state of A is a stronger base than the corresponding excited state of A, as would be expected from the fact that AH^+ is a weaker acid in the ground state. If the ground-state protonation rate constant of A (HMF or DHF) is assumed to represent the diffusion-controlled limit, the excited-state rate

TABLE 2: Calculated (AM1) Charge Densities on the Hydroxyl Oxygen [δ (O_7)]; Deprotonation (k_d) and Protonation (k_p) Rate Constants; and pK_a of 7-Hydroxy-4-methylflavylium Chloride (HMF), 4′,7-Dihydroxyflavylium Chloride (DHF), and Malvidin 3-Glucoside (Oenin), in the Ground and First Excited Singlet State

	ground state			excited state		
	HMF	DHF	Oenin	HMF[a]	DHF[b]	Oenin[c]
δ (O_7)	−0.239	−0.239	−0.235	−0.200	−0.216	−0.l87
k_d/s^{-1}	1.4×10^6	3.1×10^6	5.0×10^6	1.5×10^{11}	8.0×10^{10}	$>5 \times 10^{10}$
$k_p/10^{10}$ L mol^{-1} s^{-1}	3.56	3.06	2.51	2.3	1.9	
pK_a	4.4	4.0	3.7	−0.81	−0.62	

[a] Reference 8. [b] Reference 18. [c] The lower limit for k_d in water is assumed to be the value of 5×10^{10} s^{-1} measured in 1% aqueous methanol.[22]

constant would have to be *near-diffusion-controlled*, with a reaction efficiency of ca. $^2/_3$.[22] If this is correct, there are some interesting consequences. For instance, in the framework of the "encounter complex" approach, the rate constant for base protonation via the ($A\cdots H^+$) encounter complex would be ca. 7×10^{10} s^{-1} in the excited state ($^2/_3$ efficiency) and much higher in the ground state. Presumably, the upper limit for this rate in the ground state is dictated by the dielectric relaxation time of water (7 ps), corresponding to a maximum rate constant of 1.5×10^{11} s^{-1}. With this as the upper limit, the reaction efficiency would still be only ca. 0.8, assuming the diffusion-controlled rate to be 3.5×10^{10} L mol^{-1} s^{-1}. *This internal contradiction poses the question as to whether it is possible at all to measure diffusion-controlled rate constants with protons.* In other words, the condition $k_{reaction} \gg k_{diff}$ may never be attained because k_{diff} is large and $k_{reaction}$ must be limited by solvent reorganization.[23]

Proton Transfer in Oenin. To our knowledge, rate constants for ground state proton transfer have been published for only two natural anthocyanins: malvidin 3-glucoside (oenin)[3] and malvidin 3,5-diglucoside (malvin).[4] Those measurements, carried out with the temperature-jump technique, provided results that are 2 orders of magnitude lower than ours, both for k_d (10^4 s^{-1} range) and k_p (10^8 L mol^{-1} s^{-1} range). In our opinion, these previous values are in error because of the insufficient time resolution of the technique (>1 μs) and some peculiar features of the system. As noted above, the decay of the base form of Oenin should be a double exponential if the experimental time window covered both the nanosecond-to-microsecond (short component) and the millisecond-to-minutes (long component) ranges. Experimentally, single exponential decays are observed (see eqs 11−16 and discussion) only if the time window is restricted to either the nanosecond-to-microsecond range or the millisecond-to-minutes range. With microsecond time resolution, only the residual tail of the (nanosecond time range) decay of the base form, i.e., the transition from the fast to the slow regime, would be observable and may be strongly mixed with the longer component and noise. Obviously, from single-exponential analysis of this slice of the decay, apparent decay times much longer than those of the true short-time decay will be obtained. Thus, we suggest that these earlier measurements reflect the *transition* from the fast to slow regimes, not the fast decay itself. Both k_d and k_p must have been affected in the same way, because the experimental observable is the sum $k_d + k_p[H^+]$.

The results presented here point to at least two aspects that are of importance for understanding the acid−base behavior of natural anthocyanins in the ground and excited states. Thus, the data for Oenin demonstrate that, using our approach, one can determine the pK_a of natural anthocyanins, which typically hydrate and open to chalcones at pHs below the pK_a, i.e., for which equilibrium pK_a measurements are impossible to perform or difficult to interpret because of the competing chemistry. Unlike synthetic model flavylium salts, the acid and base forms of natural anthocyanins such as oenin fluoresce very poorly or not at all, making it very difficult (if not impossible) to demonstrate the occurrence of excited-state proton transfer in the natural anthocyanins by either steady-state or time-resolved fluorescence spectroscopy. In contrast, our results demonstrate the occurrence of efficient laser-induced perturbation of the ground-state protonation equilibrium of oenin, providing the first direct evidence that excited-state proton transfer is a significant process in natural anthocyanins.

Concluding Remarks

Deprotonation of three representative flavylium cations (two synthetic salts and a natural anthocyanin) in the ground state occurs in the submicrosecond time range, and the rate constants correlate with the charge density on the hydroxyl oxygen involved in the deprotonation. Because this charge density does not change appreciably for most anthocyanins,[21] this time range for deprotonation is expected to be typical of anthocyanins in general. Consequently, kinetic methods such as continuous-flow pH jump or temperature jump (dead times greater than 10 μs) are not applicable. The flash photolysis light-jump approach utilized in the present work thus seems to be the preferred, if not the only technique available for measurement of the kinetics of proton transfer in anthocyanins.

Protonation of the conjugate bases of flavylium cations is near-diffusion-controlled, in both the ground and excited singlet states. The term "near" reflects the fact that we do not know the exact value for diffusion control. Compared to the 10^5 fold change in k_d, the response of k_p to the large change of base strength upon electronic excitation is relatively small. This implies that the rate constant for base protonation via the ($A\cdots H^+$) encounter complex, $k_{reaction}$, is much larger than k_{diff} in both states and is ultimately limited by dielectric relaxation of the hydronium ion; that is, the protonation efficiency is always high and changes very little. A useful consequence of this is that approximate deprotonation rate constants of anthocyanins can be estimated from acidity constants and the assumption that $k_p \approx 2-3 \times 10^{10}$ L mol^{-1} s^{-1}.

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