

# Identifiability of the Model of the Intermolecular Excited-State Proton Exchange Reaction in the Presence of pH Buffer

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In this report, we describe the fluorescence kinetics and the deterministic identifiability of the intermolecular excited-state proton dissociation reaction and how the addition of pH buffer affects both. In the absence of buffer, the time-resolved fluorescence decays as a biexponential function with decay times that are invariant with pH. The information that the proton association rate in the excited state is negligible in combination with fluorescence decay traces measured at different pH, excitation, or emission wavelengths does not provide enough useful information for the unique determination of the rate constants and the spectral parameters related to absorption and emission. Hence, the model of intermolecular excited-state proton dissociation in the absence of pH buffer is not identifiable. When a pH buffer is added to this photophysical system, the proton exchange becomes reversible and the decay times now are a function of pH and buffer concentration. The deterministic identifiability analysis shows that for the unique determination of all rate constants one should collect a minimum of three fluorescence decays characterized by at least two different pH and at least two different nonzero buffer concentrations. In addition to these three traces, minimally one biexponential fluorescence trace corresponding to the pH probe in the absence of buffer has to be recorded. The requirement that at least two of these traces should be collected at the same pH, excitation, and emission wavelengths leads to unique identifiability.

## I. Introduction

Deterministic identifiability analysis deals with the determination of the parameters of a given model assuming error-free observations. There are three possible outcomes to a deterministic identifiability analysis.<sup>1,2</sup> (i) The parameters of an assumed model can be estimated uniquely, and the model is uniquely (globally) identifiable from the idealized experiment. (ii) There are a finite number of alternative estimates for some or all of the model parameters that fit the data, and the model is locally identifiable. (iii) An infinite number of model parameter estimates fit the data, and the model is unidentifiable from the experiment.

Identifiability analyses of compartmental models for excited-state processes have only rather recently been reported.<sup>3–8</sup> We have done extensive studies of the deterministic identifiability of a whole range of compartmental models with time-invariant rate constants for intermolecular as well as intramolecular two-state and three-state excited-state processes in the presence and absence of added quencher.<sup>4–7</sup> For the linear, time-invariant models, the parameters to be identified are rate constants and spectral parameters related to absorption and emission. For models with transients, we have shown that transient effects

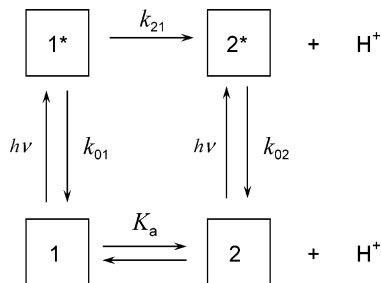
change the identifiability criteria compared to the models with time-invariant rate coefficients.<sup>8</sup>

In this paper, we describe the fluorescence kinetics and deterministic identifiability of a model of the intermolecular excited-state proton dissociation reaction. If the excited-state proton association rate is known to be negligible, the fluorescence decay surface measured at different pHs and excitation or emission wavelengths does not provide enough useful information for the unique determination of the other rate constants and the spectral parameters related to absorption and emission. The addition of a pH buffer to this photophysical system affects both its fluorescence kinetics and identifiability. Now the proton exchange reaction is reversible and the model becomes identifiable.

Deterministic identifiability not only establishes the necessary conditions for unique parameter recovery but also points the way to rational experimental design. Indeed, deterministic identifiability analysis informs us about how many fluorescence decays are needed and under which experimental conditions (excitation and emission wavelengths, pH, concentration of added buffer, etc.) they should be recorded to obtain unique parameter estimates.

On the basis of the deterministic identifiability analysis presented in this paper, time-resolved fluorescence experiments of pH indicators can be rationally planned and executed.

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**SCHEME 1: Kinetic Model of Ground- and Excited-State Proton Transfer Reactions in the Absence of pH Buffer<sup>a</sup>**


<sup>a</sup> **1** and **2** are respectively the ground-state acid–conjugate base forms of the fluorophore (fluorescent pH indicator), while **1\*** and **2\*** are the associated excited species.

Fluorescent pH indicators are widely used to investigate *in vivo* changes of proton concentrations *inside* living cells.<sup>9</sup> The intracellular pH in the cytosol is close to neutral (generally between ~6.8 and 7.4), so fluorescent indicators with a  $pK_a$  around 7 are required for cytosolic pH measurements. The most common fluorescent indicators for near-neutral pH measurements<sup>9</sup> (e.g., SNARF and SNAFL indicators,<sup>10,11</sup> BCECF<sup>11,12</sup>) are fluorescein- or rhodamine-type molecules, which can exist in several prototropic forms depending on pH. To fully understand the complex photophysics of these pH indicators, it is essential to elucidate the excited-state dynamics of these molecular entities. Rate constants of excited-state processes and spectral parameters associated with excitation and emission are the relevant parameters to be determined. The current identifiability analysis can be helpful in the rational design of time-resolved fluorescence experiments of pH indicators workable at near-neutral pH so that the right number and type of fluorescence decay traces are collected and analyzed.

## II. Model without Added pH Buffer

**A. Fluorescence Decay Kinetics.** Consider a causal, linear, time-invariant, intermolecular system consisting of two distinct types of ground-state species and two corresponding excited-state species as depicted in Scheme 1. Ground-state species **1** (acidic form of pH indicator) can deprotonate to form ground-state species **2** (conjugate base form of pH indicator) and  $H^+$ . The proton exchange reaction is characterized by the ground-state acidity constant  $K_a = [2][H^+]/[1]$  of the pH indicator. Photoexcitation creates the excited-state species **1\*** and **2\***, which can decay by fluorescence (F) and nonradiative (NR) processes. The composite rate constants for these processes are denoted by  $k_{01}$  ( $= k_{F1} + k_{NR1}$ ) and  $k_{02}$  ( $= k_{F2} + k_{NR2}$ ).  $k_{21}$  denotes the (pseudo) first-order rate constant for dissociation of **1\*** into **2\*** and  $H^+$ . Throughout this paper, we will assume that  $[H^+]$  is so small as to make the rate of the association  $2^* + H^+ \rightarrow 1^*$  negligible.

Note that we limit our study to the *classical* kinetic model of excited-state proton transfer.<sup>13</sup> It should be mentioned, however, that this *classical* kinetic model may not be the *best* description of all excited-state proton-transfer reactions. Indeed, the time evolution of excited-state proton transfer may include several processes: (i) initial formation/breaking of hydrogen bonds in the ground state, (ii) solvent reorientation and relaxation upon photoexcitation, (iii) proton dissociation, and (iv) diffusion and geminate recombination of the dissociated proton.<sup>14</sup> For a full description of all these steps encompassing subpicoseconds to several nanoseconds, a more elaborate model has to be

proposed and tested. This is beyond the scope of the current investigation.

If the system shown in Scheme 1 is excited by a  $\delta$ -pulse which does not significantly alter the concentrations of the ground-state species (i.e., in the low excitation limit), the fluorescence  $\delta$ -response function,  $f(\lambda^{em}, \lambda^{ex}, t)$ , at emission wavelength  $\lambda^{em}$  due to excitation at  $\lambda^{ex}$ , is given by<sup>4</sup>

$$f(\lambda^{em}, \lambda^{ex}, t) = \kappa \tilde{c}(\lambda^{em}) \mathbf{U} \exp(t\mathbf{\Gamma}) \mathbf{U}^{-1} \tilde{\mathbf{b}}(\lambda^{ex}) \quad t \geq 0 \quad (1)$$

with  $\kappa = (b_1 + b_2)(c_1 + c_2)$  a proportionality constant.  $\mathbf{U} \equiv [\mathbf{U}_1, \mathbf{U}_2]$  is the matrix of the two eigenvectors of the compartmental matrix  $\mathbf{A}$  (eq 2), and  $\mathbf{U}^{-1}$  is the inverse of  $\mathbf{U}$ .  $\gamma_1$  and  $\gamma_2$  are the eigenvalues of  $\mathbf{A}$  corresponding to  $\mathbf{U}_1$  and  $\mathbf{U}_2$ , respectively, and  $\exp(t\mathbf{\Gamma}) \equiv \text{diag}[\exp(\gamma_1 t), \exp(\gamma_2 t)]$ .

$$\mathbf{A} = \begin{bmatrix} -(k_{01} + k_{21}) & 0 \\ k_{21} & -k_{02} \end{bmatrix} \quad (2)$$

$\tilde{\mathbf{b}}(\lambda^{ex})$  is the  $2 \times 1$  column vector with elements  $\tilde{b}_i(\lambda^{ex})$  defined by

$$\tilde{b}_i = b_i / (b_1 + b_2) \quad (3)$$

where  $b_i$  denotes the concentration of  $i^*$  at time 0:

$$b_i = [i^*]_{t=0} \quad (4)$$

which, in the low excitation limit, is proportional to the ground-state absorbance of  $i$ . Hence, in the low excitation limit (as in single-photon timing experiments),<sup>15</sup>  $\tilde{b}_i$  represents the normalized absorbance of species  $i$  at  $\lambda^{ex}$ . The elements  $b_i$  (and  $\tilde{b}_i$ ) are dependent on pH and  $\lambda^{ex}$ .

$\tilde{c}(\lambda^{em})$  is the  $1 \times 2$  row vector of the normalized emission weighting factors  $\tilde{c}_i(\lambda^{em})$  of species  $i^*$  at  $\lambda^{em}$ .<sup>4</sup>

$$\tilde{c}_i = c_i / (c_1 + c_2) \quad (5)$$

The emission weighting factors  $c_i(\lambda^{em})$  are expressed by<sup>4</sup>

$$c_i(\lambda^{em}) = k_{F_i} \int_{\Delta\lambda^{em}} \rho_i(\lambda^{em}) d\lambda^{em} \quad (6)$$

$k_{F_i}$  represents the fluorescence rate constant of species  $i^*$ ;  $\rho_i(\lambda^{em})$  is the emission density of species  $i^*$  at emission wavelength  $\lambda^{em}$ , normalized to the complete steady-state fluorescence spectrum  $F_i$  of species  $i^*$ ; and  $\Delta\lambda^{em}$  is the emission wavelength interval around  $\lambda^{em}$  where the fluorescence signal is monitored.  $\rho_i(\lambda^{em})$  is defined by<sup>4</sup>

$$\rho_i(\lambda^{em}) = F_i(\lambda^{em}) / \int_{\text{fullband}} F_i d\lambda^{em} \quad (7)$$

Use of  $\tilde{\mathbf{b}}$  and  $\tilde{\mathbf{c}}$  in global compartmental analysis<sup>4</sup> allows one to link  $\tilde{b}_1$  (i.e.,  $\tilde{b}_1$  is considered as a single estimable parameter in the curve fitting) at the same pH and  $\lambda^{ex}$ , whereas  $\tilde{c}_1$  can be linked at the same  $\lambda^{em}$ .

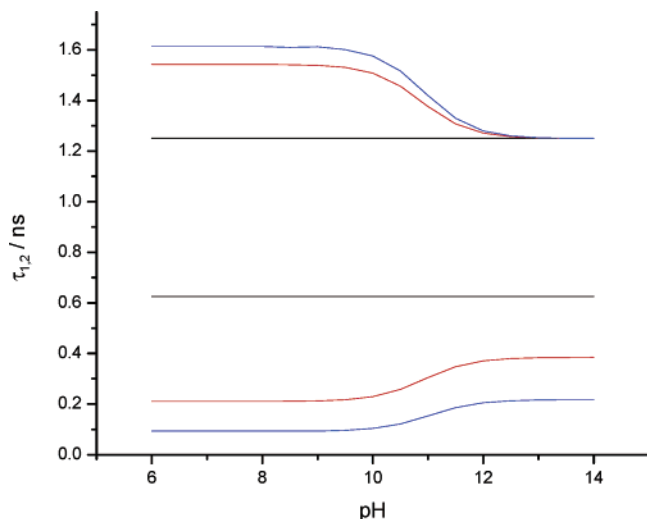
Equation 1 can be written in the common biexponential format:

$$f(\lambda^{em}, \lambda^{ex}, t) = \alpha_1 \exp(\gamma_1 t) + \alpha_2 \exp(\gamma_2 t) \quad t \geq 0 \quad (8)$$

The exponential factors  $\gamma_{1,2}$  are given by

$$\gamma_1 = -(k_{01} + k_{21}) = -S_1 \quad (9a)$$

$$\gamma_2 = -k_{02} = -S_2 \quad (9b)$$



**Figure 1.** Dependence of  $\tau_{1,2}$  on pH in the absence and presence of pH buffer (black,  $C^B = 0$  M; red,  $C^B = 0.1$  M; blue,  $C^B = 0.3$  M). The upper curves (longer lifetimes) correspond to  $\tau_2$ , the lower (shorter lifetimes) to  $\tau_1$ . The simulation values are given in Tables 1 and 2.

**TABLE 1: Simulation Values of the Parameters of the Model Depicted in Scheme 1 Used for the Calculation of  $\alpha_{1,2}$  and  $\tau_{1,2}$ <sup>a</sup>**

parameter	value	parameter	value
$k_{01}$ ( $10^9$ s <sup>-1</sup> )	0.6	$pK_a$	7.5
$k_{02}$ ( $10^9$ s <sup>-1</sup> )	0.8	$\epsilon_1/\epsilon_2$	1.0
$k_{21}$ ( $10^9$ M <sup>-1</sup> s <sup>-1</sup> )	1.0	$\tilde{c}_1$	0.0, 0.2, 0.4, 0.6

<sup>a</sup>  $\tilde{b}_1$  is calculated according to eq 56.

and are related to the decay times  $\tau_{1,2}$  according to

$$\gamma_{1,2} = -1/\tau_{1,2} \quad (10)$$

The exponential factors  $\gamma_{1,2}$  (and, hence, the decay times  $\tau_{1,2}$ ) depend exclusively on the rate constants  $k_{01}$ ,  $k_{02}$ , and  $k_{21}$  and, hence, are independent of pH (i.e.,  $[H^+]$ ). Figure 1 shows the decay times  $\tau_{1,2}$  as a function of pH. Note the invariance of  $\tau_{1,2}$  with pH when no pH buffer is present. The (arbitrary) simulation values of the parameters are compiled in Table 1. The rate constant values were chosen to obtain decay times in the (sub)nanosecond range commonly measured by the single-photon timing technique.<sup>15</sup> The other parameter values were selected to produce significant changes in the pre-exponential factors  $\alpha_{1,2}$  as a function of pH (see below).

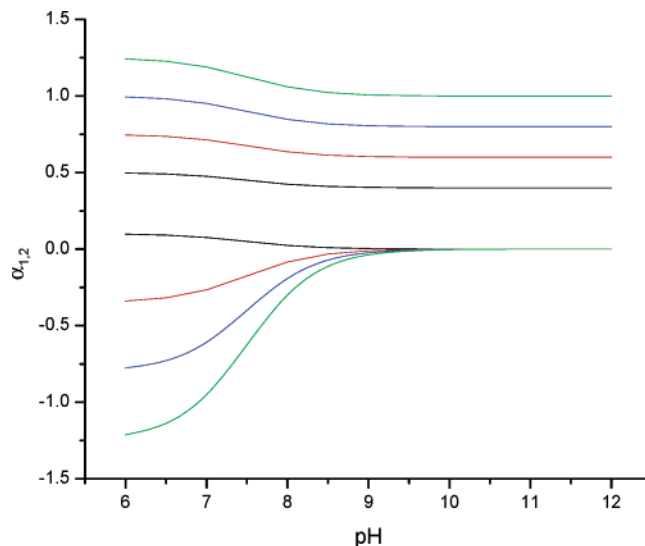
The pre-exponential factors  $\alpha_{1,2}$  are dependent on  $k_{01}$ ,  $k_{02}$ ,  $k_{21}$ ,  $\tilde{\mathbf{b}}(\lambda^{\text{ex}})$ , and  $\tilde{\mathbf{c}}(\lambda^{\text{em}})$ , as is evident from eq 11:

$$\alpha_1 = \kappa \tilde{b}_1 \left( 1 - \tilde{c}_2 - \frac{\tilde{c}_2 k_{21}}{\gamma_2 - \gamma_1} \right) \quad (11a)$$

$$\alpha_2 = \kappa \tilde{c}_2 \left( 1 - \tilde{b}_1 + \frac{\tilde{b}_1 k_{21}}{\gamma_2 - \gamma_1} \right) \quad (11b)$$

The pre-exponentials  $\alpha_{1,2}$  generally depend on pH because of the ground-state dependence of  $\tilde{\mathbf{b}}$  on  $[H^+]$ . A change of  $[H^+]$  around the numerical value of  $K_a$  will influence the ground-state composition of the pH indicator. That will be reflected in the pre-exponentials:  $\alpha_{1,2}$  will change in the pH range around  $pK_a$ . Figure 2 illustrates the pH dependence of the pre-exponentials  $\alpha_{1,2}$ .

When pH is much higher than  $pK_a$ , only species **2** and **2\*** are present and the value of the amplitude  $\alpha_1$  associated with



**Figure 2.** Dependence of  $\alpha_{1,2}$  on pH in the absence of buffer, corresponding to different  $\tilde{c}_1$  values (black,  $\tilde{c}_1 = 0.6$ ; red,  $\tilde{c}_1 = 0.4$ ; blue,  $\tilde{c}_1 = 0.2$ ; green,  $\tilde{c}_1 = 0.0$ ). The upper curves correspond to  $\alpha_2$ , the lower to  $\alpha_1$ . The simulation values are those of Table 1.

the limiting value of  $\tau_1 = (k_{01} + k_{21})^{-1} = S_1^{-1}$  vanishes ( $\tilde{b}_1 = 0$ ; see Figure 2). Hence, a monoexponential decay is obtained (eq 12),

$$f(\lambda^{\text{em}}, \lambda^{\text{ex}}, t) = b_2 c_2 \exp(-k_{02} t) \quad t \geq 0 \quad (12)$$

and this assigns a unique value to  $k_{02}$ . In combination with a biexponential decay, we can thus assign unique values to  $S_1 = k_{01} + k_{21}$  and  $S_2 = k_{02}$  (see section II.B). Equation 12 presumes that  $b_2 \neq 0$  and  $c_2 \neq 0$  (otherwise no fluorescence would be observed).

On the other hand, when only the acidic form of the pH indicator is excited and its fluorescence monitored ( $\tilde{b}_1 = 1$  and  $\tilde{c}_1 = 1$ ), a monoexponential decay is found (eq 13):

$$f(\lambda^{\text{em}}, \lambda^{\text{ex}}, t) = b_1 c_1 \exp(-S_1 t) \quad t \geq 0 \quad (13)$$

The condition  $\tilde{c}_1 = 1$  implies that  $k_{01} \neq 0$  ( $c_1$  contains  $k_{F1}$ , see eq 6).

Another limiting case is when  $\tilde{b}_1 = 1$  and  $\tilde{c}_1 = 0$  (excite exclusively the acidic form of the pH probe and monitor the emission of its basic form only). In this case, a dual exponential decay is obtained (eq 14)

$$f(\lambda^{\text{em}}, \lambda^{\text{ex}}, t) = \frac{b_1 c_2 k_{21}}{\gamma_2 - \gamma_1} [-\exp(-S_1 t) + \exp(-k_{02} t)] \quad t \geq 0 \quad (14)$$

The condition here is that  $k_{21} \neq 0$ .

**B. Deterministic Identifiability.** An outstanding way of formulating the problem of identifiability is whether one can find different realizations of  $f(t)$ , say  $(\mathbf{A}, \mathbf{b}, \mathbf{c})$  and  $(\mathbf{A}^+, \mathbf{b}^+, \mathbf{c}^+)$ , so that<sup>2,7</sup>

$$f(t, \mathbf{A}, \mathbf{b}, \mathbf{c}) = f(t, \mathbf{A}^+, \mathbf{b}^+, \mathbf{c}^+) \quad (15)$$

that is, the fluorescence  $\delta$ -response function is the same. Unique identifiability of the model is obtained when  $\mathbf{A}^+ = \mathbf{A}$ ,  $\mathbf{b}^+ = \mathbf{b}$ , and  $\mathbf{c}^+ = \mathbf{c}$ . If a limited number of alternative  $\mathbf{A}^+$ ,  $\mathbf{b}^+$ , and  $\mathbf{c}^+$  exist, the model is locally identifiable. If the number of  $\mathbf{A}^+$ ,  $\mathbf{b}^+$ , and  $\mathbf{c}^+$  is infinite, the model is said to be unidentifiable. One way of constructing another realization  $(\mathbf{A}^+, \mathbf{b}^+, \mathbf{c}^+)$  of

$f(t)$  is via similarity transformation,<sup>2,7</sup> giving

$$\mathbf{A}^+ = \mathbf{T}^{-1}\mathbf{A}\mathbf{T} \quad (16)$$

where  $\mathbf{T}$  is a constant invertible (or nonsingular) matrix (i.e.,  $\det \mathbf{T} \neq 0$ ) having the same dimension as  $\mathbf{A}$

$$\mathbf{T} = \begin{bmatrix} t_1 & t_2 \\ t_3 & t_4 \end{bmatrix} \quad (17)$$

One can rewrite eq 16 in the form

$$\mathbf{T}\mathbf{A}^+ = \mathbf{A}\mathbf{T} \quad (18)$$

The alternative  $\mathbf{b}^+$  and  $\mathbf{c}^+$  are given by<sup>7</sup>

$$\mathbf{b}^+ = \mathbf{T}^{-1}\mathbf{b} \quad (19a)$$

$$\mathbf{c}^+ = \mathbf{c}\mathbf{T} \quad (19b)$$

The main advantage of the method of similarity transformation is that it not only shows if a model is identifiable or not but it also gives the precise relationship between the true and alternative model parameters.

Equations 16 (or 18) and 19 should be satisfied for each experimental condition [different pH, analytical buffer concentration  $C^B$  (see section III), and excitation and emission wavelengths  $\lambda^{\text{ex}}$  and  $\lambda^{\text{em}}$ ]. This implies that matrix  $\mathbf{T}$  should be independent of pH,  $C^B$ ,  $\lambda^{\text{ex}}$ , and  $\lambda^{\text{em}}$ . Indeed, because  $\mathbf{c}^+$  should not depend on pH,  $C^B$ , and  $\lambda^{\text{ex}}$ ,  $\mathbf{T}$  should be independent of pH,  $C^B$ , and  $\lambda^{\text{ex}}$ . Similarly, because of the independence of  $\mathbf{b}^+$  (and  $\mathbf{A}^+$ ) of  $\lambda^{\text{em}}$ ,  $\mathbf{T}$  should be independent of  $\lambda^{\text{em}}$ .

Performing the matrix multiplication in eq 18 yields

$$-t_1(k_{01}^+ + k_{21}^+ - k_{01} - k_{21}) + t_2k_{21}^+ = 0 \quad (20a)$$

$$-t_2(k_{02}^+ - k_{01} - k_{21}) = 0 \quad (20b)$$

$$t_1k_{21} + t_3(k_{01}^+ + k_{21}^+ - k_{02}) - t_4k_{21}^+ = 0 \quad (20c)$$

$$t_2k_{21} + t_4(k_{02}^+ - k_{02}) = 0 \quad (20d)$$

A nontrivial solution (not all of  $t_1, \dots, t_4$  are 0) to the homogeneous system (eq 20) of four linear equations in four unknowns  $t_1, \dots, t_4$  is found when the fourth-order determinant of the matrix of the coefficients of the unknowns equals 0. This leads to the following condition:

$$(k_{01}^+ + k_{21}^+ - k_{01} - k_{21})(k_{02}^+ - k_{01} - k_{21})(k_{01}^+ + k_{21}^+ - k_{02})(k_{02}^+ - k_{02}) = 0 \quad (21)$$

Equation 21 is fulfilled when  $k_{02}^+ = k_{02}$ . From eq 20d with  $t_4 \neq 0$ , we have that  $t_2 = 0$ , which leads from eq 20a with  $t_1 \neq 0$  to  $k_{01}^+ + k_{21}^+ = k_{01} + k_{21}$ . If  $t_2 = 0$ , the original system is obtained and the matrix  $\mathbf{T}$  takes the form

$$\mathbf{T} = \begin{bmatrix} t_1 & 0 \\ t_3 & t_4 \end{bmatrix} \quad (22)$$

with  $t_1 \neq 0$  and  $t_4 \neq 0$  ( $\mathbf{T}$  is nonsingular). The similarity transformation shows that only values for  $S_2 = k_{02}$  and  $S_1 = k_{01} + k_{21}$  can be obtained.

From eq 20b with  $t_2 \neq 0$ , we have that  $k_{02}^+ = k_{01} + k_{21}$  and, thus, eq 21 is fulfilled. If we then substitute  $k_{02}^+ = k_{01} + k_{21}$  in eq 20, we obtain  $k_{01}^+ + k_{21}^+ = k_{02}$  as a solution.

To summarize, two sets of rate constant values are obtained: set I is the original set:  $k_{01}^+ + k_{21}^+ = k_{01} + k_{21}$  and  $k_{02}^+ = k_{02}$  with  $\mathbf{T}$  given by eq 22; set II is the alternative:  $k_{01}^+ + k_{21}^+ = k_{02}$  and  $k_{02}^+ = k_{01} + k_{21}$  is just the interchanged solution for  $S_2 (= k_{02})$  and  $S_1$ . Thus, the model is locally identifiable in terms of  $S_1$  and  $S_2$  but is unidentifiable in terms of the individual rate constants.

For set I, the alternative  $\mathbf{b}^+$  and  $\mathbf{c}^+$  are calculated from eqs 19 with  $\mathbf{T}$  given by eq 22:

$$b_1^+ = b_1/t_1 \quad (23a)$$

$$b_2^+ = (-t_3b_1/t_1 + b_2)/t_4 \quad (23b)$$

$$c_1^+ = t_1c_1 + t_3c_2 \quad (23c)$$

$$c_2^+ = t_4c_2 \quad (23d)$$

Because  $t_1, t_3$ , and  $t_4$  are all nonzero, there are an infinite number of alternative  $\mathbf{b}^+$  and  $\mathbf{c}^+$ . It is straightforward to demonstrate that there also is an infinite number of alternative  $\mathbf{b}^+$  and  $\mathbf{c}^+$  for set II (calculated according to eqs 19 with  $\mathbf{T}$  given by eq 17). To conclude, the model of intermolecular excited-state proton dissociation in the absence of pH buffer is not identifiable.

In the remainder of this section we use an alternative approach to identifiability, which can give the number and type of decays that are needed for identifiability. We will discuss whether the parameters  $k_{01}, k_{02}, k_{21}, \bar{\mathbf{b}}$ , and  $\bar{\mathbf{c}}$  can uniquely be determined by algebraic manipulations of  $\gamma_i$  and  $\alpha_i$  ( $i = 1, 2$ ), the descriptive parameters of  $f(\lambda^{\text{em}}, \lambda^{\text{ex}}, t)$  obtained at different known pH values, and at various excitation and emission wavelengths. Therefore, the aim of this deterministic identifiability approach is to investigate whether it is possible to derive expressions for the parameters  $k_{ij}, \bar{\mathbf{b}}$ , and  $\bar{\mathbf{c}}$  as a function of  $\gamma_i$  and  $\alpha_i$  (which are assumed to be exactly known).

The first set of equations is generated by the elementary symmetric functions  $\sigma_i$  ( $i = 1, 2$ ) in  $\gamma_i$  ( $i = 1, 2$ ) defined by<sup>2</sup>

$$\sigma_1 \equiv \gamma_1 + \gamma_2 \quad (24a)$$

$$\sigma_2 \equiv \gamma_1\gamma_2 \quad (24b)$$

The functions  $\sigma_{1,2}$  are functions of  $k_{ij}$  only. The explicit expressions corresponding to eq 24 are given by

$$\sigma_1 \equiv -k_{01} - k_{21} - k_{02} = -S_1 - S_2 \quad (25a)$$

$$\sigma_2 \equiv S_1S_2 \quad (25b)$$

Because  $\sigma_1$  and  $\sigma_2$  are symmetric functions in  $S_1$  and  $S_2 (= k_{02})$ , eq 25 has interchangeable solutions for  $S_1$  and  $k_{02} (= S_2)$ :

$$S_1 = -\frac{1}{2}(\sigma_1 - \sqrt{\sigma_1^2 - 4\sigma_2}) \quad \text{and} \quad k_{02} = -\frac{1}{2}(\sigma_1 + \sqrt{\sigma_1^2 - 4\sigma_2}) \quad (26a)$$

and

$$S_1 = -\frac{1}{2}(\sigma_1 + \sqrt{\sigma_1^2 - 4\sigma_2}) \quad \text{and} \quad k_{02} = -\frac{1}{2}(\sigma_1 - \sqrt{\sigma_1^2 - 4\sigma_2}) \quad (26b)$$

Therefore, on the basis of the decay times of a single decay

trace, there are two possibilities to assign numerical values to  $S_1$  and  $S_2$  ( $=k_{02}$ ). This is in complete agreement with the results from the identifiability based on similarity transformation. As mentioned before, the pre-exponentials as a function of pH can resolve this ambiguity. Indeed, for  $[H^+] \rightarrow 0$  (i.e., high pH), only the pre-exponential factor  $\alpha_2$  associated with  $k_{02}$  remains and  $f(t)$  becomes monoexponential (eq 12). Hence, if a decay trace at high pH can be recorded, unique numerical values can be assigned to  $k_{02}$  and  $S_1$ . However, it remains impossible to assign unique values to the individual rate constants  $k_{01}$  and  $k_{21}$  when no buffer is added.

The second set of equations is provided by the Markov parameters  $m_i$  which can be expressed as a function of  $\mathbf{A}$ ,  $\tilde{\mathbf{b}}$ , and  $\tilde{\mathbf{c}}^2$

$$m_i = \kappa \tilde{\mathbf{c}} \mathbf{A}^i \tilde{\mathbf{b}}, \quad i = 0, 1 \quad (27)$$

For a biexponential  $f(t)$  (eq 8), the Markov parameters can be expressed as a function of the pre-exponentials  $\alpha_i$  and eigenvalues  $\gamma_i$

$$m_i = \sum_{j=1}^2 \alpha_j \gamma_j^i, \quad i = 0, 1 \quad (28)$$

with  $\gamma_j^i$  being the  $i$ th power of  $\gamma_j$ . The explicit expressions for  $m_0$  and  $m_1$  are

$$m_0 = \kappa(\tilde{b}_1 + \tilde{c}_2 - 2\tilde{b}_1\tilde{c}_2) \quad (29a)$$

$$m_1 = \kappa\{[-(1 - \tilde{c}_2)(k_{01} + k_{21}) + \tilde{c}_2 k_{21}]\tilde{b}_1 - \tilde{c}_2 k_{02}(1 - \tilde{b}_1)\} \quad (29b)$$

$m_0$  and  $m_1$  depend on pH only through the ground-state equilibrium. Furthermore,  $m_0$  and  $m_1$  also are dependent on  $\lambda^{\text{ex}}$  and  $\lambda^{\text{em}}$ . Elimination of the scaling factor  $\kappa$  from  $m_0$  and  $m_1$  yields eq 30:

$$\tilde{b}_1[(m_0 k_{21} + P + Q)\tilde{c}_2 - P] - Q\tilde{c}_2 = 0 \quad (30)$$

with

$$P = m_1 + m_0(k_{01} + k_{21}) = m_1 + m_0 S_1 \quad (31a)$$

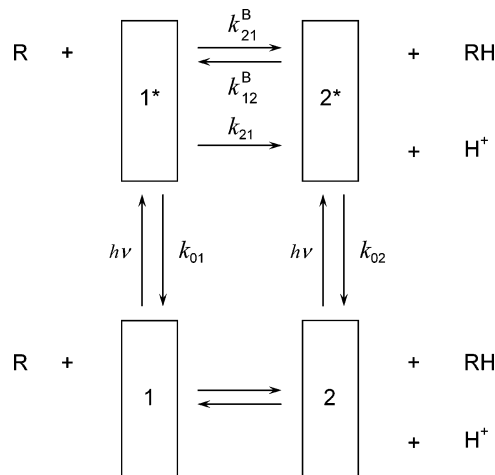
$$Q = m_1 + m_0 k_{02} \quad (31b)$$

$P$  and  $Q$  generally depend on pH,  $\lambda^{\text{ex}}$ , and  $\lambda^{\text{em}}$ . Note that eq 30 is a homogeneous nonlinear equation in three unknowns, namely,  $k_{21}$ ,  $\tilde{b}_1$ , and  $\tilde{c}_2$ . Equation 30 has a trivial solution for  $\tilde{b}_1 = 0$ ,  $\tilde{c}_2 = 0$ , and  $k_{21}$  of any value. If the trivial solution is excluded, dividing eq 30 by  $\tilde{b}_1\tilde{c}_2$  converts it into a linear equation in three unknowns ( $k_{21}$ ,  $1/\tilde{b}_1$ , and  $1/\tilde{c}_2$ ):

$$m_0 k_{21} - (P/\tilde{c}_2) - (Q/\tilde{b}_1) + (P + Q) = 0 \quad (32)$$

For a single decay trace collected at a given pH and a single emission wavelength  $\lambda^{\text{em}}$  due to excitation at  $\lambda^{\text{ex}}$ , eq 30 or eq 32 provides the basic equation from which the values of  $k_{21}$ ,  $\tilde{b}_1$ , and  $\tilde{c}_2$  must be derived. For the model to be identifiable from a single decay trace, two out of three unknowns ( $k_{21}$ ,  $\tilde{b}_1$ , and  $\tilde{c}_2$ ) have to be known beforehand. Therefore, we can conclude that, in the absence of any a priori information, the model of intermolecular excited-state proton dissociation in the absence of pH buffer is not identifiable. Additional equations

## SCHEME 2: Kinetic Model of Ground- and Excited-State Proton Exchange Reactions in the Presence of pH Buffer<sup>a</sup>



<sup>a</sup> RH and R are respectively the acid and conjugate base forms of the buffer. The other symbols are as in Scheme 1.

constructed at different excitation/emission wavelengths are linearly dependent with eq 32<sup>6</sup> no matter which excitation or emission wavelengths are used.

### III. Model with Added pH Buffer

**A. Fluorescence Decay Kinetics.** Now we will consider the system with added pH buffer (with analytical concentration  $C^B$ ) as depicted in Scheme 2. The acidity of the buffer can be described by its ground-state acidity constant  $K_a^B$ :

$$K_a^B = \frac{[R][H^+]}{[RH]} \quad (33)$$

In the ground state, species **1** can react with the conjugate base form R of the pH buffer to give species **2** and RH (acidic form of the buffer). The reaction is in principle reversible. In the excited state, the reaction of species **1\*** with R to form **2\*** and RH is characterized by the rate constant  $k_{21}^B$ . The reverse reaction of **2\*** and RH to give **1\*** and R is described by the rate constant  $k_{12}^B$ . The meanings of the other rate constants  $k_{ij}$  are as in Scheme 1.

The fluorescence  $\delta$ -response function,  $f(\lambda^{\text{em}}, \lambda^{\text{ex}}, t)$ , at emission wavelength  $\lambda^{\text{em}}$  due to excitation at  $\lambda^{\text{ex}}$ , is given by eq 1 with  $\mathbf{A}$  given by eq 34.

$$\mathbf{A} = \begin{bmatrix} -(k_{01} + k_{21} + k_{21}^B[R]) & k_{12}^B[RH] \\ k_{21} + k_{21}^B[R] & -(k_{02} + k_{12}^B[RH]) \end{bmatrix} \quad (34)$$

Equation 1 can be written in the common dual exponential format (eq 8) with  $\gamma_{1,2}$  given by

$$\gamma_{1,2} = -\frac{1}{2}\{(S_1 + S_2) \mp [(S_1 - S_2)^2 + 4k_{12}^B[RH](k_{21} + k_{21}^B[R])]^{1/2}\} \quad (35)$$

with

$$S_1 = k_{01} + k_{21} + k_{21}^B[R] \quad (36a)$$

$$S_2 = k_{02} + k_{12}^B[RH] \quad (36b)$$

**TABLE 2: Simulation Values of the Parameters of the Model Depicted in Scheme 2 Used for the Calculation of  $\alpha_{1,2}$  and  $\tau_{1,2}$ <sup>a</sup>**

parameter	value	parameter	value
$k_{01}$ ( $10^9$ s <sup>-1</sup> )	0.6	$pK_a$	7.5
$k_{02}$ ( $10^9$ s <sup>-1</sup> )	0.8	$pK_a^B$	10.64
$k_{21}$ ( $10^9$ M <sup>-1</sup> s <sup>-1</sup> )	1.0	$\epsilon_1/\epsilon_2$	1.0
$k_{12}^B$ ( $10^9$ M <sup>-1</sup> s <sup>-1</sup> )	30.0	$\tilde{c}_1$	0.0, 0.2, 0.4, 0.6
$k_{21}^B$ ( $10^9$ M <sup>-1</sup> s <sup>-1</sup> )	10.0		

<sup>a</sup>  $\tilde{b}_1$  is calculated according to eq 56.

In contrast to the case without added pH buffer, the exponential factors  $\gamma_{1,2}$  (and  $\tau_{1,2}$ ) now depend on pH (and  $K_a^B$  and  $C^B$ ) because [R] and [RH] are pH-dependent.

Indeed, [R] and [RH] can be expressed as a function of  $[H^+]$ ,  $K_a^B$ , and the buffer concentration  $C^B$  ( $= [R] + [RH]$ ):

$$[R] = \frac{K_a^B C^B}{K_a^B + [H^+]} \quad (37a)$$

$$[RH] = \frac{[H^+] C^B}{K_a^B + [H^+]} \quad (37b)$$

Figure 1 illustrates the pH dependence of  $\tau_{1,2}$ . The simulation values of the parameters are compiled in Table 2.

The pre-exponentials  $\alpha_{1,2}$  are dependent on  $k_{ij}$ ,  $\mathbf{b}$ ,  $\mathbf{c}$ , pH, and  $C^B$  (through [R] and [RH]),

$$\alpha_1 = c_1 \beta_{11} + c_2 \beta_{21} \quad (38a)$$

$$\alpha_2 = c_1 \beta_{12} + c_2 \beta_{22} \quad (38b)$$

with

$$\beta_{11} = \{b_1(S_2 + \gamma_1) + b_2 k_{12}^B [RH]\} / (\gamma_1 - \gamma_2) \quad (38c)$$

$$\beta_{12} = \{b_1(S_1 + \gamma_1) - b_2 k_{12}^B [RH]\} / (\gamma_1 - \gamma_2) \quad (38d)$$

$$\beta_{21} = \{b_1(k_{21} + k_{21}^B [R]) + b_2(S_1 + \gamma_1)\} / (\gamma_1 - \gamma_2) \quad (38e)$$

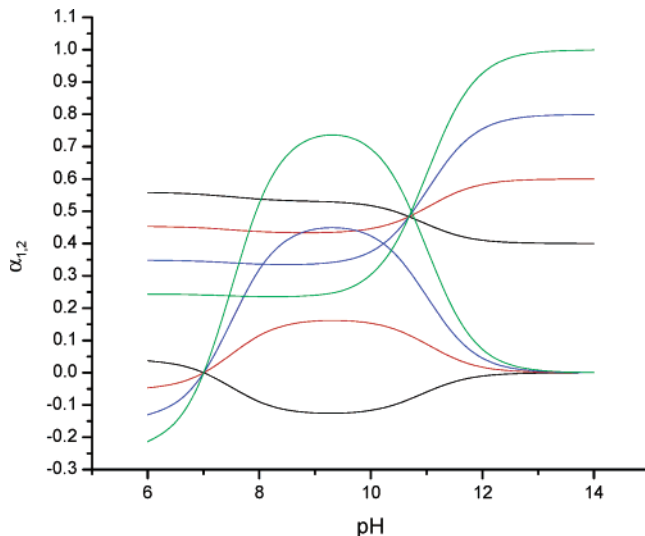
$$\beta_{22} = \{-b_1(k_{21} + k_{21}^B [R]) + b_2(S_2 + \gamma_1)\} / (\gamma_1 - \gamma_2) \quad (38f)$$

Because  $S_1 + \gamma_1 = -S_2 - \gamma_2$ , there are alternative ways to write eq 38c–f. Figure 3 displays  $\alpha_{1,2}$  as a function of pH. The pre-exponential factors vary most clearly around  $pK_a$  and  $pK_a^B$ .

When pH is much higher than  $pK_a$  and  $pK_a^B$ , only species **2** and **2\*** are present and  $[RH] \approx 0$  ( $C^B \approx [R]$ ). In this case, the value of the amplitude  $\alpha_1$  associated with the limiting value of  $\tau_1 = (k_{01} + k_{21} + k_{21}^B C^B)^{-1} = S_1^{-1}$  vanishes and the fluorescence  $\delta$ -response function is given by eq 12, just as in the case without buffer (see Figure 3).

**B. Deterministic Identifiability.** Now we will discuss whether adding pH buffer to the system (as depicted in Scheme 2) can lead to a globally identifiable model, that is, to the unique determination of the rate constants  $k_{01}$ ,  $k_{02}$ ,  $k_{21}$ ,  $k_{21}^B$ , and  $k_{12}^B$  and the spectral parameters  $\tilde{\mathbf{b}}$  and  $\tilde{\mathbf{c}}$ .

To investigate if there are alternative realizations of  $f(t)$ , namely,  $(\mathbf{A}^+, \mathbf{b}^+, \mathbf{c}^+)$ , fulfilling eq 15, we calculate the matrix



**Figure 3.** Dependence of  $\alpha_{1,2}$  on pH in the presence of 0.1 M pH buffer, corresponding to different  $\tilde{c}_1$  values (black,  $\tilde{c}_1 = 0.6$ ; red,  $\tilde{c}_1 = 0.4$ ; blue,  $\tilde{c}_1 = 0.2$ ; green,  $\tilde{c}_1 = 0.0$ ). The upper curves correspond to  $\alpha_1$ , the lower to  $\alpha_2$ . The simulation values are compiled in Table 2.

multiplication given by eq 18 with  $\mathbf{A}$  given by eq 34 and  $\mathbf{T}$  expressed by eq 17.

Performing the matrix multiplication in eq 18 yields

$$-t_1(k_{01}^+ + k_{21}^+ - k_{01} - k_{21}) + t_2 k_{21}^+ = (t_1 k_{21}^{B+} - t_1 k_{21}^B - t_2 k_{21}^{B+})[R] + t_3 k_{12}^B [RH] \quad (39a)$$

$$-t_2(k_{02}^+ - k_{01} - k_{21}) = -t_2 k_{21}^B [R] + (-t_1 k_{12}^{B+} + t_2 k_{12}^{B+} + t_4 k_{12}^B)[RH] \quad (39b)$$

$$-t_1 k_{21} - t_3(k_{01}^+ + k_{21}^+ - k_{02}) + t_4 k_{21}^+ = (t_1 k_{21}^B + t_3 k_{21}^{B+} - t_4 k_{21}^{B+})[R] - t_3 k_{12}^B [RH] \quad (39c)$$

$$-t_2 k_{21} - t_4(k_{02}^+ - k_{02}) = t_2 k_{21}^B [R] + (-t_3 k_{12}^{B+} + t_4 k_{12}^{B+} - t_4 k_{12}^B)[RH] \quad (39d)$$

Because the elements  $t_i$  of  $\mathbf{T}$  are independent of [R] and [RH], the coefficients of [R] and [RH] have to be 0, leading to

$$t_2 = 0 \quad (40a)$$

$$t_3 = 0 \quad (40b)$$

$$t_1(k_{21}^{B+} - k_{21}^B) = 0 \quad (40c)$$

$$-t_1 k_{12}^{B+} + t_4 k_{12}^B = 0 \quad (40d)$$

$$t_1 k_{21}^B - t_4 k_{21}^{B+} = 0 \quad (40e)$$

$$t_4(k_{12}^{B+} - k_{12}^B) = 0 \quad (40f)$$

From eq 40c we have that  $k_{21}^{B+} = k_{21}^B$ , because the alternative ( $t_1 = 0$ ) leads to  $t_4 = 0$ . In combination with eq 40e, we have that  $t_1 = t_4$ . From eq 40f, we have that  $k_{12}^{B+} = k_{12}^B$ , because the alternative ( $t_4 = 0$ ) leads to  $t_1 = 0$ . In combination with eq 40d, we also have that  $t_1 = t_4$ . Parts a, c, and d of eq 39

can be rewritten as

$$-t_1(k_{01}^+ + k_{21}^+ - k_{01} - k_{21}) = 0 \quad (41a)$$

$$t_1(k_{21}^+ - k_{21}) = 0 \quad (41b)$$

$$t_1(k_{02}^+ - k_{02}) = 0 \quad (41c)$$

From eq 41c, it follows that  $k_{02}^+ = k_{02}$ . From eq 41b, we have that  $k_{21}^+ = k_{21}$ , and in combination with eq 41a we obtain  $k_{01}^+ = k_{01}$ . So there is only one set of rate constants possible: the alternative set of rate constants equals the original set ( $k_{ij}^+ = k_{ij}$ ) and is obtained when  $\mathbf{T}$  takes the form

$$\mathbf{T} = t_1 \begin{bmatrix} 1 & 0 \\ 0 & 1 \end{bmatrix} = t_1 \mathbf{I} \quad (42)$$

with  $t_1 \neq 0$  and  $\mathbf{I}$  being the  $2 \times 2$  identity matrix.

Substituting eq 42 in eq 19 yields the alternative  $\mathbf{b}^+$  and  $\mathbf{c}^+$ :

$$\mathbf{b}^+ = \mathbf{b}/t_1 \quad (43a)$$

$$\mathbf{c}^+ = t_1 \mathbf{c} \quad (43b)$$

Because  $t_1$  is not accessible in time-resolved fluorescence experiments, it is more appropriate to formulate the identifiability problem in terms of normalized  $\tilde{\mathbf{b}}$ ,  $\tilde{\mathbf{c}}$ , and the proportionality factor  $\kappa = (b_1 + b_2)(c_1 + c_2)$  (eq 1). Indeed, use of normalized  $\tilde{\mathbf{b}}$  and  $\tilde{\mathbf{c}}$  in global compartmental analysis<sup>4</sup> allows  $\tilde{b}_1$  to be linked at the same pH and  $\lambda^{\text{ex}}$ , whereas  $\tilde{c}_1$  can be linked at the same  $\lambda^{\text{em}}$ .

Therefore, we try to find different realizations of  $f(t)$ , say  $(\mathbf{A}, \tilde{\mathbf{b}}, \tilde{\mathbf{c}}, \kappa)$  and  $(\mathbf{A}, \tilde{\mathbf{b}}^+, \tilde{\mathbf{c}}^+, \kappa^+)$ , so that

$$f(t, \mathbf{A}, \tilde{\mathbf{b}}, \tilde{\mathbf{c}}, \kappa) = f(t, \mathbf{A}^+, \tilde{\mathbf{b}}^+, \tilde{\mathbf{c}}^+, \kappa^+) \quad (44)$$

The model is uniquely identifiable when  $\mathbf{A}^+ = \mathbf{A}$ ,  $\tilde{\mathbf{b}}^+ = \tilde{\mathbf{b}}$ ,  $\tilde{\mathbf{c}}^+ = \tilde{\mathbf{c}}$ , and  $\kappa^+ = \kappa$ . Substituting the spectral parameters (eq 43) yields

$$\tilde{\mathbf{b}}^+ = \tilde{\mathbf{b}} \quad (45a)$$

$$\tilde{\mathbf{c}}^+ = \tilde{\mathbf{c}} \quad (45b)$$

$$\kappa^+ = \kappa \quad (45c)$$

Because  $\mathbf{A}^+ = \mathbf{A}$ ,  $\tilde{\mathbf{b}}^+ = \tilde{\mathbf{b}}$ ,  $\tilde{\mathbf{c}}^+ = \tilde{\mathbf{c}}$ , and  $\kappa^+ = \kappa$ , the model of intermolecular excited-state proton exchange in the presence of pH buffer is uniquely identifiable.

It must be emphasized that  $[\mathbf{R}]$  and  $[\mathbf{RH}]$  in eqs 39 can be expressed in terms of  $K_a^{\text{B}}$ ,  $[\text{H}^+]$ , and  $C^{\text{B}}$  via eq 37. Because the elements  $t_i$  of  $\mathbf{T}$  must be independent of  $[\text{H}^+]$  and  $C^{\text{B}}$ , this leads to eq 40 and, thus, to the same identifiability conditions as derived above.

The similarity transformation is an extremely powerful approach to identifiability because it establishes a direct relationship between the original  $(\mathbf{A}, \tilde{\mathbf{b}}, \tilde{\mathbf{c}}, \kappa)$  and the alternative  $(\mathbf{A}, \tilde{\mathbf{b}}^+, \tilde{\mathbf{c}}^+, \kappa^+)$  system parameters (via eqs 18 and 19) and demonstrates directly if a model is identifiable or not. However, it does not tell which experimental design will lead to identifiability. In other words, although similarity transformation excels in informing us if a model is identifiable, it does not reveal how many fluorescence decays are necessary and under which experimental conditions (pH, buffer concentration,  $\lambda^{\text{ex}}$ , and  $\lambda^{\text{em}}$ ) they should be recorded to achieve identifiability.

Therefore, we turn now to the identifiability method based on  $\sigma$  functions and Markov parameters to learn about the experimental conditions under which identifiability of the model of Scheme 2 will be realized.

$\sigma_1$  and  $\sigma_2$  are functions of  $k_{ij}$ ,  $[\mathbf{R}]$ , and  $[\mathbf{RH}]$ , and are explicitly expressed by

$$\sigma_1 = -(k_{01} + k_{21} + k_{02}) - (k_{21}^{\text{B}}[\mathbf{R}] + k_{12}^{\text{B}}[\mathbf{RH}]) \quad (46a)$$

$$\sigma_2 = k_{02}(k_{01} + k_{21}) + k_{02}k_{21}^{\text{B}}[\mathbf{R}] + k_{01}k_{12}^{\text{B}}[\mathbf{RH}] \quad (46b)$$

Equation 46 can be rewritten as a function of the acidity constant  $K_a^{\text{B}}$  of the buffer and the two independent experimental variables  $[\text{H}^+]$  and  $C^{\text{B}}$ :

$$\sigma_1 = -(k_{01} + k_{21} + k_{02}) - C^{\text{B}} \frac{k_{21}^{\text{B}}K_a^{\text{B}} + k_{12}^{\text{B}}[\text{H}^+]}{K_a^{\text{B}} + [\text{H}^+]} \quad (47a)$$

$$\sigma_2 = k_{02}(k_{01} + k_{21}) + C^{\text{B}} \frac{k_{02}k_{21}^{\text{B}}K_a^{\text{B}} + k_{01}k_{12}^{\text{B}}[\text{H}^+]}{K_a^{\text{B}} + [\text{H}^+]} \quad (47b)$$

To indicate the dependence of  $\sigma_1$  and  $\sigma_2$  on buffer concentration  $C^{\text{B}}$  and pH, we will use the notations  $\sigma_{1ki}$  and  $\sigma_{2ki}$  to represent respectively  $\sigma_1$  and  $\sigma_2$  at buffer concentration  $C_k^{\text{B}}$  and  $\text{pH}_i$  (corresponding to  $[\text{H}^+]_i$ ).

A biexponential decay trace in the absence of pH buffer ( $C^{\text{B}} = 0$ ) yields values of  $k_{02}$  and the sum  $(k_{01} + k_{21})$ . This information [the sum  $(k_{01} + k_{21} + k_{02})$ ] combined with two decays in the presence of buffer measured at two pHs allows one to determine unique values of  $k_{21}^{\text{B}}$  and  $k_{12}^{\text{B}}$ . From  $\sigma_{1k1}$  and  $\sigma_{1k2}$  at two pHs and a common buffer concentration  $C_k^{\text{B}}$  we have:

$$k_{21}^{\text{B}} = \{\sigma_{1k1}[\text{H}^+]_2(K_a^{\text{B}} + [\text{H}^+]_1) - \sigma_{1k2}[\text{H}^+]_1(K_a^{\text{B}} + [\text{H}^+]_2) - K_a^{\text{B}}([\text{H}^+]_1 - [\text{H}^+]_2)(k_{01} + k_{21} + k_{02})\} / \{C_k^{\text{B}}K_a^{\text{B}}([\text{H}^+]_1 - [\text{H}^+]_2)\} \quad (48a)$$

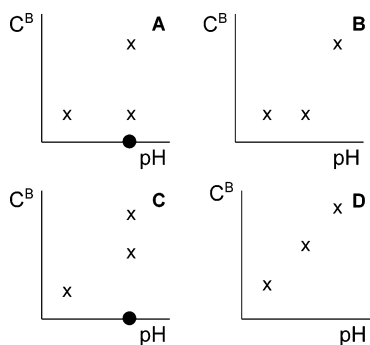
$$k_{12}^{\text{B}} = \{-\sigma_{1k1}(K_a^{\text{B}} + [\text{H}^+]_1) + \sigma_{1k2}(K_a^{\text{B}} + [\text{H}^+]_2) - ([\text{H}^+]_1 - [\text{H}^+]_2)(k_{01} + k_{21} + k_{02})\} / \{C_k^{\text{B}}([\text{H}^+]_1 - [\text{H}^+]_2)\} \quad (48b)$$

In fact, the buffer concentration does not have to be identical for the two pHs. Just two pHs are necessary and sufficient for the unique determination of  $k_{21}^{\text{B}}$  and  $k_{12}^{\text{B}}$ . Indeed, from  $\sigma_{1k1}$  at  $\text{pH}_1$  and  $C_k^{\text{B}}$  and  $\sigma_{1k2}$  at  $\text{pH}_2$  and  $C_l^{\text{B}}$ , we have

$$k_{21}^{\text{B}} = \{\sigma_{1k1}[\text{H}^+]_2C_l^{\text{B}}(K_a^{\text{B}} + [\text{H}^+]_1) - \sigma_{1k2}[\text{H}^+]_1C_k^{\text{B}}(K_a^{\text{B}} + [\text{H}^+]_2) + (k_{01} + k_{21} + k_{02})\{C_l^{\text{B}}[\text{H}^+]_2(K_a^{\text{B}} + [\text{H}^+]_1) - C_k^{\text{B}}[\text{H}^+]_1(K_a^{\text{B}} + [\text{H}^+]_2)\}\} / \{C_k^{\text{B}}C_l^{\text{B}}K_a^{\text{B}}([\text{H}^+]_1 - [\text{H}^+]_2)\} \quad (49a)$$

$$k_{12}^{\text{B}} = \{-\sigma_{1k1}C_l^{\text{B}}(K_a^{\text{B}} + [\text{H}^+]_1) + \sigma_{1k2}C_k^{\text{B}}(K_a^{\text{B}} + [\text{H}^+]_2) + (k_{01} + k_{21} + k_{02})\{C_k^{\text{B}}(K_a^{\text{B}} + [\text{H}^+]_2) - C_l^{\text{B}}(K_a^{\text{B}} + [\text{H}^+]_1)\}\} / \{C_k^{\text{B}}C_l^{\text{B}}([\text{H}^+]_1 - [\text{H}^+]_2)\} \quad (49b)$$

To summarize, once the sum  $(k_{01} + k_{21} + k_{02})$  is known (from a biexponential decay of the pH indicator without added



**Figure 4.** Possible experimental configurations as a function of pH and buffer concentration  $C^B$  that lead to uniquely determined rate constants. All four cases depend on the knowledge of the sum  $(k_{01} + k_{21} + k_{02})$ , which can be obtained from a biexponential decay without pH buffer ( $C^B = 0$ ). Moreover, the experimental configurations A and C give a globally identifiable model (in terms of  $k_{ij}$ ,  $b$ , and  $\bar{c}$ ) if the decays measured at the common pH [two nonzero values of  $C^B$  (x) and one at  $C^B = 0$  (●)] are recorded under identical experimental conditions ( $\lambda^{ex}$  and  $\lambda^{em}$ ).

pH buffer), two pHs are necessary and sufficient to determine unique values of  $k_{21}^B$  and  $k_{12}^B$  from the  $\sigma_1$  functions. Note that the knowledge of  $(k_{01} + k_{21} + k_{02})$  combined with decays at two different buffer concentrations and the same pH (using  $\sigma_{1k1}$  at  $C_k^B$  and  $\sigma_{1l1}$  at  $C_l^B$ ) does not lead to a solution of  $k_{21}^B$  and  $k_{12}^B$ .

Figure 4 illustrates the four experimental configurations as a function of pH and  $C^B$ , which lead to the unique determination of all rate constants ( $k_{01}$ ,  $k_{21}$ ,  $k_{02}$ ,  $k_{21}^B$ , and  $k_{12}^B$ ). Figure 4A shows the situation with two pHs and two nonzero values of  $C^B$ . In Figure 4B, three different pHs and two nonzero values of  $C^B$  are used. Figure 4C depicts the configuration with two pHs and three nonzero values of  $C^B$ . Finally, three pH and three nonzero values of  $C^B$  also lead to the unique determination of  $k_{ij}$  (Figure 4D). Of course, each of the four configurations of Figure 4 can be represented somewhat differently. As long as at least two pHs and at least two nonzero values of  $C^B$  are used in combination with a biexponential decay at  $C^B = 0$ , one should obtain unique values for  $k_{ij}$ . Mathematically, the system of two  $\sigma_1$  and three  $\sigma_2$  equations (eq 47) was used to obtain unique solutions for the five rate constants  $k_{ij}$  via a symbolic mathematics program. For all configurations in Figure 4, the knowledge of the sum  $(k_{01} + k_{21} + k_{02})$  is essential to obtain unique solutions of all rate constants. Indeed, without knowledge of  $(k_{01} + k_{21} + k_{02})$ , five eqs 47 at different  $C^B$  and pH as shown in Figure 4 yield unique values of  $k_{21}^B$  and  $k_{12}^B$ , while  $k_{01}$ ,  $k_{21}$ , and  $k_{02}$  are roots of quadratic equations. One can say that in that case the model is locally identifiable in terms of the rate constants  $k_{ij}$ . If one wants unique solutions for all five rate constants, one should include a dual exponential decay in the absence of buffer.

It should be mentioned that, in the absence of a priori information, three different pHs and a common buffer concentration do not produce a solution for  $k_{01}$ ,  $k_{21}$ , and  $k_{02}$ . Conversely, three decay curves at the same pH but different buffer concentrations do not lead to the determination of any rate constant, even if  $(k_{01} + k_{21})$  and  $k_{02}$  are known. However, in the limiting case when the pH of the solution is much smaller than the  $pK_a^B$  of the buffer ( $pH \ll pK_a^B$ ), one has that the buffer exists practically only under its acidic form ( $[RH] \approx C^B$ ). In that case,  $k_{12}^B$  can be determined from  $\sigma_1$  at a single buffer concentration and pH, if the sum  $(k_{01} + k_{21} + k_{02})$  is known from a biexponential decay of the pH indicator at  $C^B = 0$ . If

additionally  $(k_{01} + k_{21})$  and  $k_{02}$  are separately known,  $k_{01}$  and  $k_{12}^B$  can be determined from  $\sigma_1$  and  $\sigma_2$  at a single buffer concentration and pH.

We just showed that time-resolved data (i.e.,  $\sigma_1$  values) at two different pHs are necessary and sufficient for the determination of  $k_{21}^B$  and  $k_{12}^B$  if the sum  $(k_{01} + k_{21} + k_{02})$  is known. This presumes the measurement of a biexponential decay of the pH indicator at  $C^B = 0$ . In the next paragraph, we will investigate what rate constant information can be extracted from decays of the pH indicator as a function of pH only (i.e., at a single buffer concentration). This information is useful for systems where only the pH is varied at a single buffer concentration.<sup>16</sup>

If the only known information about the rate constants is the value of  $k_{02}$  (e.g., from the monoexponential decay at high pH in the presence or absence of pH buffer), the four remaining rate constants ( $k_{01}$ ,  $k_{21}$ ,  $k_{21}^B$ , and  $k_{12}^B$ ) cannot be determined from  $\sigma_{1k1}$ ,  $\sigma_{1k2}$ ,  $\sigma_{2k1}$ , and  $\sigma_{2k2}$  at two pHs (1 and 2) and a common buffer concentration  $C_k^B$ . However, if in addition to  $k_{02}$  the sum  $(k_{01} + k_{21})$  is known, unique values for  $k_{01}$  (or  $k_{21}$ ),  $k_{21}^B$ , and  $k_{12}^B$  can be determined from two  $\sigma_1$  values and one  $\sigma_2$  value (or one  $\sigma_1$  value and two  $\sigma_2$  values) at two pHs and a single buffer concentration. Similarly, if both  $k_{01}$  and  $k_{02}$  are known, the other rate constants ( $k_{21}$ ,  $k_{21}^B$ , and  $k_{12}^B$ ) can be uniquely determined at two pHs. However, when  $k_{21}$  and  $k_{02}$  are both known,  $k_{01}$ ,  $k_{21}^B$ , and  $k_{12}^B$  are the roots of quadratic equations, resulting in two possible solutions for each of these rate constants. This ambiguity in the rate constants can possibly be resolved if some of the rate constants assume negative values.

In the above analyses, we used  $\sigma_{1,2}$  equations (eq 47) written as a function of  $K_a^B$  and the experimentally independent variables  $[H^+]$  and  $C^B$ . One might wonder if one also can do the identifiability analysis using eq 46, where  $\sigma_{1,2}$  is expressed as a function of  $[R]$  and  $[RH]$ . Because five rate constants ( $k_{01}$ ,  $k_{21}$ ,  $k_{02}$ ,  $k_{21}^B$ , and  $k_{12}^B$ ) have to be determined, one needs three fluorescence decays producing three  $\sigma_1$  and three  $\sigma_2$  equations. For the first decay trace, we can choose the experimental variables  $C_1^B \neq 0$  and  $[H^+]_1$  in principle at random as long as a clear biexponential function is obtained. This decay allows us to construct  $\sigma_{1,2}$  as a function of  $[R]_1$  and  $[RH]_1$  (eq 46). The choice of experimental  $C_2^B$  and  $[H^+]_2$  for the second decay is limited to cases where  $C_2^B \neq C_1^B$  or  $[H^+]_2 \neq [H^+]_1$ . The second decay produces  $\sigma_{1,2}$  as a function of  $[R]_2$  and  $[RH]_2$  (eq 46). For the third decay, however, the experimental variables  $C_3^B$  and  $[H^+]_3$  cannot be chosen freely if unique solutions of the five rate constants are to be found. Indeed, two ( $[R]_3$ ,  $[RH]_3$ ) concentrations give unique solutions of  $k_{ij}$ : (i)  $[R]_3 = [R]_2$  and  $[RH]_3 = [RH]_1$  and (ii)  $[R]_3 = [R]_1$  and  $[RH]_3 = [RH]_2$ . The first requirement leads to the experimental variables  $[H^+]_3$  and  $C_3^B$  given by eq 50:

$$[H^+]_3 = \frac{C_1^B [H^+]_1 (K_a^B + [H^+]_2)}{C_2^B (K_a^B + [H^+]_1)} \quad (50a)$$

$$C_3^B = \frac{C_2^B (K_a^B + [H^+]_3)}{(K_a^B + [H^+]_2)} \quad (50b)$$

The second (symmetrical) condition gives the experimental variables  $[H^+]_3$  and  $C_3^B$  of eq 51:



$$[\text{H}^+]_3 = \frac{C_2^{\text{B}}[\text{H}^+]_2(K_a^{\text{B}} + [\text{H}^+]_1)}{C_1^{\text{B}}(K_a^{\text{B}} + [\text{H}^+]_2)} \quad (51a)$$

$$C_3^{\text{B}} = \frac{C_1^{\text{B}}(K_a^{\text{B}} + [\text{H}^+]_3)}{K_a^{\text{B}} + [\text{H}^+]_1} \quad (51b)$$

Equations 50 and 51 are symmetrical (the subscripts 1 and 2 are exchanged). These two conditions produce similar configurations as shown in Figure 4, but now  $[\text{H}^+]_3$  and  $C_3^{\text{B}}$  of the third decay cannot be chosen freely.

To summarize, four configurations (Figure 4) lead to unique solutions of the five rate constants under two conditions. (i) The independent experimental variables  $[\text{H}^+]$  and  $C^{\text{B}}$  can be chosen freely for three decays at nonzero  $C^{\text{B}}$ , but then a biexponential decay at  $C^{\text{B}} = 0$  has to be added. (ii) The alternative does not require a decay curve without added buffer. The experimental variables  $[\text{H}^+]$  and  $C^{\text{B}}$  ( $\neq 0$ ) of two decays can be selected freely, but then  $[\text{H}^+]_3$  and  $C_3^{\text{B}}$  of the third decay have to fulfill eqs 50 or 51.

Once values of the rate constants  $k_{ij}$  are known, we will investigate now the conditions for the unique determination of the spectral parameters  $\tilde{b}_1$  and  $\tilde{c}_1$ .

The expression for  $m_0$  is given by eq 29a,  $m_1$  is given by eq 52:

$$m_1 = \kappa \{ \tilde{b}_1 [\tilde{c}_2 (k_{21} + k_{12}^{\text{B}}[\text{R}] + S_1 + S_2) - S_1 - k_{12}^{\text{B}}[\text{RH}] - \tilde{c}_2 (S_2 + k_{12}^{\text{B}}[\text{RH}]) + k_{12}^{\text{B}}[\text{RH}] \} \quad (52)$$

with  $S_1$  and  $S_2$  given by eq 36.  $m_0$  and  $m_1$  depend on pH,  $C^{\text{B}}$ ,  $\lambda^{\text{ex}}$ , and  $\lambda^{\text{em}}$ . Elimination of the scaling factor  $\kappa$  from  $m_0$  and  $m_1$  yields eq 53:

$$\tilde{b}_1 \{ [m_0 (k_{21} + k_{12}^{\text{B}}[\text{R}] + P + Q) \tilde{c}_2 - P - m_0 k_{12}^{\text{B}}[\text{RH}] \} - \tilde{c}_2 (Q + m_0 k_{12}^{\text{B}}[\text{RH}]) + m_0 k_{12}^{\text{B}}[\text{RH}] = 0 \quad (53)$$

with

$$P = m_1 + m_0 S_1 \quad (54a)$$

$$Q = m_1 + m_0 S_2 \quad (54b)$$

Equation 53 can be rewritten as a function of the independent experimental variables  $[\text{H}^+]$  (i.e., pH) and  $C^{\text{B}}$ :

$$\begin{aligned} & -\tilde{b}_1 \tilde{c}_2 \{ m_1 + m_0 (k_{01} + 2k_{21} + k_{02}) + \\ & 2m_0 C^{\text{B}} (k_{21}^{\text{B}} K_a^{\text{B}} + k_{12}^{\text{B}}[\text{H}^+]) / (K_a^{\text{B}} + [\text{H}^+]) \} + \\ & \tilde{b}_1 \{ m_1 + m_0 (k_{01} + k_{21}) + m_0 C^{\text{B}} (k_{21}^{\text{B}} K_a^{\text{B}} + k_{12}^{\text{B}}[\text{H}^+]) / \\ & (K_a^{\text{B}} + [\text{H}^+]) \} + \tilde{c}_2 \{ m_1 + m_0 k_{02} + 2m_0 C^{\text{B}} k_{12}^{\text{B}}[\text{H}^+]/ \\ & (K_a^{\text{B}} + [\text{H}^+]) \} = m_0 C^{\text{B}} k_{12}^{\text{B}}[\text{H}^+] / (K_a^{\text{B}} + [\text{H}^+]) \quad (55) \end{aligned}$$

Equation 55 is a nonhomogeneous equation in two unknowns,  $\tilde{b}_1$  and  $\tilde{c}_2$ . To create another equation of type 55 with the same  $\tilde{b}_1$  and  $\tilde{c}_2$ , one should collect a decay trace at the same pH and  $\lambda^{\text{ex}}$  (i.e., same  $\tilde{b}_1$ ) and identical  $\lambda^{\text{em}}$  (i.e., same  $\tilde{c}_2$ ) but at a different nonzero buffer concentrations  $C^{\text{B}}$ . The solution to such a system of two eqs 55 in two unknowns ( $\tilde{b}_1$ ,  $\tilde{c}_2$ ) results in two solutions for  $\tilde{b}_1$  and  $\tilde{c}_2$ . If the biexponential decay at  $C^{\text{B}} = 0$  is recorded at the same pH,  $\lambda^{\text{ex}}$ , and  $\lambda^{\text{em}}$  as for eq 55, one can use eq 30 together with eq 55 to obtain values for  $\tilde{b}_1$  and  $\tilde{c}_2$ . Also here two solutions are obtained for  $\tilde{b}_1$  and  $\tilde{c}_2$ . The constraints

( $0 \leq \tilde{b}_1, \tilde{c}_2 \leq 1$ ) may lead to unique values for the spectral parameters. The system of three eqs 55 obtained at three different nonzero values of  $C^{\text{B}}$  and the same pH,  $\lambda^{\text{ex}}$ , and  $\lambda^{\text{em}}$  always leads to a unique solution of  $\tilde{b}_1$  and  $\tilde{c}_2$ . The same is true for the system of two eqs 55 (at two nonzero values of  $C^{\text{B}}$ ) and one eq 30 (at  $C^{\text{B}} = 0$ ). Hence, the situations depicted in Figure 4A,C lead to global identifiability in terms of  $k_{ij}$ ,  $\tilde{\mathbf{b}}$ , and  $\tilde{\mathbf{c}}$ . The requirement in Figure 4A,C is that the decay traces at the common pH should be collected under identical experimental conditions ( $\lambda^{\text{ex}}$  and  $\lambda^{\text{em}}$ ).

Finally, when some knowledge about the photophysical system can be assessed through other (i.e., not time-resolved fluorescence) experiments, it might be useful in obtaining global identifiability with less fluorescence decays than discussed above. For instance, the  $\tilde{\mathbf{b}}$  parameters can be expressed as a function of the ground-state acidity constant  $K_a$  of the pH indicator, the molar absorption coefficients  $\epsilon_i(\lambda^{\text{ex}})$  of ground-state species  $i$  at  $\lambda^{\text{ex}}$ , and the pH of the sample solution. For  $\tilde{\mathbf{b}}_{1k}$  at  $[\text{H}^+]_k$  and  $\lambda^{\text{ex}}$  we have

$$\frac{1}{\tilde{b}_{1k}} = 1 + \frac{\epsilon_2(\lambda^{\text{ex}})K_a}{\epsilon_1(\lambda^{\text{ex}})[\text{H}^+]_k} \quad (56)$$

The  $\tilde{b}_{1k}$  values calculated according to eq 56 can then be used in the analysis and possibly reduce the number of fluorescence decays needed for unique identifiability.

#### IV. Conclusions

In this paper, we have derived the equations describing the fluorescence decay kinetics of the intermolecular excited-state proton transfer reaction in the absence and presence of added pH buffer. When the proton association reaction in the excited state is negligible, the decay times are invariant with pH in the absence of buffer. The assumption that the association rate constant equals 0 for the investigated system is not helpful in determining unique values for the remaining parameters. It is shown that only the values of  $k_{01}$  and the sum ( $k_{01} + k_{21}$ ) can be obtained in that case. The deterministic identifiability analysis shows that additional decay curves measured at different pH, excitation, or emission wavelengths do not provide enough independent information useful for the unique determination of the remaining parameters. Addition of buffer results in a reversible excited-state proton transfer reaction and yields decay times that become pH-dependent. To uniquely determine all rate constants  $k_{ij}$ , a minimum of three fluorescence decay traces should be collected for the pH probe in the presence of buffer. These three decays should be characterized by at least two different pHs and at least two different nonzero buffer concentrations. In addition to these three traces, minimally one trace corresponding to the pH probe without buffer has to be recorded for the unique determination of all rate constants  $k_{ij}$ . The requirement that the decays at the common pH should be collected under identical experimental conditions ( $\lambda^{\text{ex}}$  and  $\lambda^{\text{em}}$ ) leads to unique identifiability (in terms of  $k_{ij}$ ,  $\tilde{\mathbf{b}}$ , and  $\tilde{\mathbf{c}}$ ).

To investigate the deterministic identifiability we have used two approaches: (i) the standard approach,<sup>1,2,4-6</sup> based on equations involving Markov parameters and  $\sigma_{1,2}$  functions, and (ii) the similarity transformation method,<sup>2,7</sup> which provides direct relations between the true and alternative sets of parameters  $k_{ij}$ ,  $\tilde{\mathbf{b}}$ , and  $\tilde{\mathbf{c}}$ . Use of normalized  $\tilde{b}$  and  $\tilde{c}$  in global compartmental analysis allows  $\tilde{b}_1$  to be linked at the same pH and  $\lambda^{\text{ex}}$ , whereas  $\tilde{c}_1$  can be linked at the same  $\lambda^{\text{em}}$ .

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