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TIME RESOLVED SPECTROSCOPY OF TRYPTOPHYL FLUORESCENCE OF YEAST 3-PHOSPHOGLYCERATE KINASE

Jean-Paul PRIVAT, Philippe WAHL and Jean-Claude AUCHET
Centre de Biophysique Moléculaire, C.N.R.S., 45045 Orleans cedex, France

and

Roger H. PAIN
Department of Biochemistry, University of New-Castle upon Tyne, Newcastle upon Tyne NE1 7RU, UK

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The tryptophyl fluorescence emission of yeast 3-phosphoglycerate kinase decreases from pH 3.9 to pH 7.2 following a normal titration curve with an apparent pK of 4.7. The fluorescence decays have been determined at both extreme pH by photocounting pulse fluorimetry and have been found to vary with the emission wavelength. A quantitative analysis of these results according to a previously described method allows to determine the emission characteristics of the two tryptophan residues present in the protein molecule. At pH 3.9, one of the tryptophan residues is responsible for only 13% of the total fluorescence emission. This first residue has a lifetime $\tau_1 = 0.6$ ns and a maximum fluorescence wavelength $\lambda_{1max} = 332$ nm. The second tryptophan residue exhibits two lifetimes $\tau_{21} = 3.1$ ns and $\tau_{22} = 7.0$ ns ($\lambda_{2max} = 338$ nm). In agreement with the attribution of τ_{21} and τ_{22} to the same tryptophan residue, the ratio $\beta = C_{21}/C_{22}$ of the normalized amplitudes is constant along the fluorescence emission spectrum. At pH 7.2, the two tryptophan residues contribute almost equally to the protein fluorescence. The decay time of tryptophan 1 is 0.4 ns. The other emission parameters are the same as those determined at pH 3.9. We conclude that the fluorescence quenching in the range pH 3.9 to pH 8.0 comes essentially from the formation of a non emitting internal ground state complex between the tryptophan having the longest decay times and a neighbouring protein chemical group. The intrinsic pK of this group and the equilibrium constant of the internal complex can be estimated. The quenching group is thought to be a carboxylate anion. Excitation transfers between the two tryptophyl residues of the protein molecule appear to have a small efficiency.

1. Introduction

The emission of protein solutions, irradiated at 295 nm, is essentially due to the indole chromophore. Since the physical properties of this emission are highly sensitive to environment, tryptophan residues have been used as reporter groups of protein conformation changes caused by ligand binding, solvent perturbation or temperature increase. This is the reason why much work has been done in order to correlate physical and chemical perturbations with spectroscopic properties of indole. In spite of these numerous studies, two difficulties are still encountered in protein fluorescence.

First, even for simple indole derivatives, the correlation between spectral properties and solvation are far from definitively established (for review see Lumry and Hershberger [1]).

Secondly, most proteins contain more than one tryptophan residue. Their fluorescence spectrum is a composite one as a result of the different environment of each tryptophan residue. In preceding works we have been able to separate the fluorescence spectra of two different tryptophyl emissions by determining the fluorescence decays as a function of emission wavelength. For example, it has been possible to reconstitute the individual spectra of the folded and unfolded conformers of cyclo–glycyl and cyclo–alanyl tryptophan [2]. More recently, the spectra of the two individual tryptophan residues of the lac repressor protein of E. coli have been reconstituted and their opposite

wavelength shift in presence of the inducer I.P.T.G. have been shown up [3].

In the work reported here, we applied the same method to the study of yeast 3-phosphoglycerate kinase (P.G.K.). This enzyme of the glycolytic pathway is a very attractive one since its monomer is made of two lobes joined together by a "waist" of diameter 2 nm [4]. A similar structure has been found in yeast hexokinase [5], adenylate kinase [6], horse muscle phosphoglycerate kinase [7] and is expected to have some relation with the function of these enzymes. It has recently been shown that the enzyme fluorescence is a function of pH [8].

By using time resolved spectroscopy, we have been able to decompose the tryptophyl fluorescence spectrum into two components which have been attributed to each of the two tryptophan residues. In addition we found that the fluorescence decrease which is observed when the pH increases from 3.9 to 7 was due to a static quenching of one of the residues. The equilibrium constants of this interaction have been evaluated.

2. Materials and methods

The enzyme was isolated as previously described (9) or purchased from Boehringer Mannheim Co. Absorption spectra and optical densities were measured with a Beckman Acta III spectrophotometer. The protein concentration was determined by absorbance measurements at 280 nm using $A^{280}_{1\%} = 4.9$ [10,11].

2.1. Fluorimetric titration

The pH measurements were performed with a Tacussel pH meter equipped with a TCBC 11/HS/SM glass electrode. The pH of a solution of P.G.K. in 0.1 M NaCl was adjusted with small aliquots of diluted NaOH or HCl. In order to avoid protein denaturation, each addition was made slowly with a gentle stirring of the protein solution. Any increase in pH did not exceed 0.1 unity. The pH was measured before and after the fluorescence experiments in order to check the solution stability. No pH change was found to occur during the fluorescence measurements, even near the isoionic point of the protein.

2.2. Steady state fluorescence measurements

Fluorescence spectra were measured with a Jobin-Yvon type Beam spectrofluorimeter modified in our laboratory. The apparatus was equipped with a XBO-150 W xenon lamp, two prism monochromators and a 6256 B EMI photomultiplier. Fluorescence quantum yields of P.G.K. were measured relatively to N-acetyl-tryptophanamide in distilled water. Their determinations were made according to the method described by Demas and Crosby [12]. Emission spectra were corrected for photomultiplier sensitivity and prism dispersion. The quantum yields (Φ) were calculated according to the following equation:

$$\Phi_p = \Phi_s \times \frac{A_p}{A_s} \times 10^{(OD_p - OD_s)/2} \times \frac{OD_s}{OD_p} \qquad (1)$$

where s and p refer to the standard and protein solution respectively and where the symbols OD and A designate the optical densities at the excitation wavelength and the area under the fluorescence spectra of the samples.

2.3. Fluorescence titration analysis

In order to take into account the results of the fluorescence decay, we were led to explain the fluorescence change with pH by the following reaction scheme

$$PH \rightleftharpoons P^- + H^+ \rightleftharpoons P{-}TR^- \qquad (2)$$

where PH, P^-, $P{-}TR^-$ are respectively the acid form and two ionized forms of the protein molecule. In the $P{-}TR^-$ form, the ionized group (presumably a carboxylate) comes in contact with one of the two tryptophan residues and quenches completely its fluorescence.

One defines the following equilibrium constants:

$$K_1 = [P^-][H^+]/[PH], \qquad (3)$$

$$K_2 = [P{-}TR^-]/[P^-], \qquad (4)$$

$$K_a = K_1(1+K_2) = [H^+]\{[P^-] + [P{-}TR^-]\}/[PH]. \qquad (5)$$

K_1 and K_a are respectively the intrinsic and apparent dissociation constant of the carboxylic acid whereas K_2 is an isomerization constant.

The fluorescence intensity of the three protein molecule species present in the solution will be designated

by F_{PH}, F_{P^-}, F_{P-TR^-} and their quantum yield by q_{PH}, q_{P^-}, q_I. We assume that ionization by itself does not affect the protein quantum yield. Static quenching occurs only after the isomerization reaction. Hence we define $q_{PH} = q_{P^-} = q_{II}$. In the fluorescent state I, one of the tryptophan residues interacts with a quencher group whereas in state II this interaction does not occur.

We can then write:

$$F_{PH} = q_{II}[PH] = q_{II} \times \frac{[P_0]}{1+K_a/[H^+]},$$

where $[P_0]$ is the total molar concentration in protein.

$$F_{P^-} = q_{II}[P^-] = \frac{q_{II}[P_0]}{1+K_2} \times \frac{K_a/[H^+]}{1+K_a/[H^+]},$$

$$F_{P-TR^-} = q_I[P-TR^-] = \frac{q_I[P_0]K_2}{1+K_2} \times \frac{K_a/[H^+]}{1+K_a/[H^+]}.$$

The whole fluorescence emission F is the sum of these three contributions. For $K_a/[H^+] \gg 1$, the intensity becomes:

$$F_{min} = \frac{(q_{II} + q_I K_2)[P_0]}{1+K_2}.$$

When $K_a/[H^+] \ll 1$, the intensity reaches:

$$F_{max} = q_{II}[P_0]$$

It can readily be shown that F varies with pH according to a classical titration curve, the equation of which can be written as follows:

$$\log \frac{F_{max}-F}{F-F_{min}} = pH + \log K_a, \quad (7)$$

where F_{max} and F_{min} are respectively the maximum and the minimum intensities corresponding to the high and low pH plateaus. The ratio of these two intensities is given by:

$$\frac{F_{max}}{F_{min}} = \frac{1+K_2}{1+(q_I/q_{II})K_2}. \quad (8)$$

If we assume that the radiative rate constant is identical for the two tryptophan residues, one can write:

$$q_I/q_{II} = \langle\tau_I\rangle/\langle\tau_{II}\rangle, \quad (9)$$

where $\langle\tau_I\rangle$ and $\langle\tau_{II}\rangle$ are the mean decay times of the protein fluorescence in the states I and II.

In the particular case of yeast 3-phosphoglycerate kinase, two fluorescence transitions overlap in the acidic pH range. So that, while F_{min} can be measured directly, F_{max} had to be estimated. This was done by extrapolating the plot $1/(F-F_{min})$ versus $1/[H^+]$ at $1/[H^+] = 0$.

2.4. Principle of the method of the time resolved spectroscopy

As the general principle of the method has already been described [13] we will only give here formulae adapted to the present study. We consider a protein containing two tryptophan residues. Let us assume that the fluorescence decay at a given emission wavelength is the sum of three exponential functions as follows:

$$I(\lambda,t) = C_1(\lambda)\exp(-t/\tau_1) + C_{21}(\lambda)\exp(-t/\tau_{21}) + C_{22}(\lambda)\exp(-t/\tau_{22}), \quad (12)$$

τ_1 is the decay time characterizing the tryptophan residue number 1, τ_{21} and τ_{22} the residue number 2. The fluorescence spectra of the two chromophores are different but the two decay times τ_{21} and τ_{22} are associated with the same spectrum. This means that the ratio $C_{21}(\lambda)/C_{22}(\lambda)$ does not depend on the emission wavelength. The whole fluorescence spectrum of the protein is the sum of two spectra each arising from one of the two emitters. One can write:

$$F(\lambda) = F_1(\lambda) + F_2(\lambda),$$

and obtain the two resolved spectra by the following expressions:

$$F_1(\lambda) = \frac{C_1(\lambda)\tau_1}{C_1(\lambda)\tau_1 + C_{21}(\lambda)\tau_{21} + C_{22}(\lambda)\tau_{22}} \times F(\lambda) \quad (13)$$

$$F_2(\lambda) = \frac{C_{21}(\lambda)\tau_{21} + C_{22}(\lambda)\tau_{22}}{C_1(\lambda)\tau_1 + C_{21}(\lambda)\tau_{21} + C_{22}(\lambda)\tau_{22}} \times F(\lambda) \quad (14)$$

Furthermore the areas S_1 and S_2 under the fluorescence spectra $F_1(\lambda)$ are related to the molar concentration $[A_1]$ and $[A_2]$ of each of the two residues by the following relation:

$$\frac{S_1}{S_2} \times \frac{\langle\tau_2\rangle}{\tau_1} = \frac{k_{F_1}\epsilon_1}{k_{F_2}\epsilon_2} \times \frac{[A_1]}{[A_2]}, \quad (15)$$

where the average lifetime $\langle\tau_2\rangle$ is given by:

$$\langle\tau_2\rangle = \frac{C_{21}(\lambda)\tau_{21} + C_{22}(\lambda)\tau_{22}}{C_{21}(\lambda) + C_{22}(\lambda)}$$

for any value of the emission wavelength λ. k_{F_1}, ϵ_1, k_{F_2}, ϵ_2 are the radiative rate constant of deactivation and the molar absorptivity for residue 1 and 2 respectively.

2.5. Fluorescence decay measurements

The transient fluorescences excited by a short light pulse were measured using a photoelectron counting apparatus described earlier. The excitation light pulse was generated by a free running flash lamp operating in hydrogen/neon mixtures (Auchet and Wahl, unpublished work) with a frequency at 10KHz. The excitation wavelength was selected by a Bausch and Lomb 250 mm monochromator with different bandwidths. Fluorescence emission wavelength was selected through a 500 mm Bausch and Lomb monochromator with a bandwidth generally set at 3 nm. It was verified that under these optical conditions the straylight was negligible. Photons were collected on a RCA 8 850 photomultiplier.

The response function $g(t)$ of the apparatus was obtained with a solution of para-terphenyl in cyclohexane as already described [14] ($\tau = 0.96$ ns). Measurements with the reference compound were made before and after each observation of the protein solutions. This procedure allowed us to test the reproducibility of the apparatus response function.

The parameters of the sum of exponentials which fits the measured transient fluorescence were obtained by a computing program where the reference transient fluorescence was directly introduced [15,16]. The quality of the fit was assessed by using the residual R defined by the following expression:

$$R = \frac{1}{n}\sum_{i=1}^{n}(i_c^k - i_{ex}^k)^2/(i_{ex}^k),$$

where i_{ex}^k and i_c^k are the number of counts in the kth channel of the experimental and the calculated curves respectively. The program repeated systematically the calculation of R with different sets of time constants τ_i increased by a fixed increment. The set of τ_i which led to the minimum of R was selected.

The amplitudes (pre-exponential terms) were determined, using the moments of the studied transient fluorescence and of the reference, by a set of linear equations which take into account the truncature of the data [17]. When the sample fluorescence was fitted with a three time constant decay, it was necessary to use moments until the third order. The fit of the best parameters obtained were also evaluated by examining the deviation functions D_v^k and the precision parameter P [15] defined by:

$$D_v^k = (i_c^k - i_{ex}^k)/(i_{ex}^k)^{1/2}, \qquad P = ((R-1)/M_0)^{1/2},$$

where M_0 is the moment of order zero of the fluorescence decay curve.

The three time constants were assumed to be the same for all the sample fluorescence of a series obtained at fixed emission wavelength and various excitation wavelengths or at fixed excitation wavelength and various emission wavelengths. A modified version of the computing program outlined above was then written which computed $S = \Sigma_{j=1}^{n} P_j$ for a given set of time constants, where P_j was the precision parameter of the sample fluorescence measured at the variable wavelength λ_j and n the number of variable wavelengths chosen. This program selected the set of time constants which minimizes S. The simultaneous analysis of a series of fluorescence data was also carried out by a third type of program where the ratio of the amplitude of the second and the third exponential terms (C_{21}/C_{22}) was set equal to a given value β. For this purpose, in the set of linear equations which leads to the amplitude values, the third equation involving the moment of order three was replaced by the relation $C_{21} - \beta C_{22} = 0$.

3. Results

3.1. Steady-state fluorescence measurements

The pH dependence of the fluorescence of yeast 3-phosphoglycerate kinase is shown in fig. 1. Experiments were carried out in NaCl 0.1 M at 20°C. Excitation wavelength was set at 295 nm. In these measurements, we used the intensity at 340 nm which can be considered as proportional to the quantum yield since the fluorescence spectrum is only slightly shifted along the pH range explored. In agreement with a previous

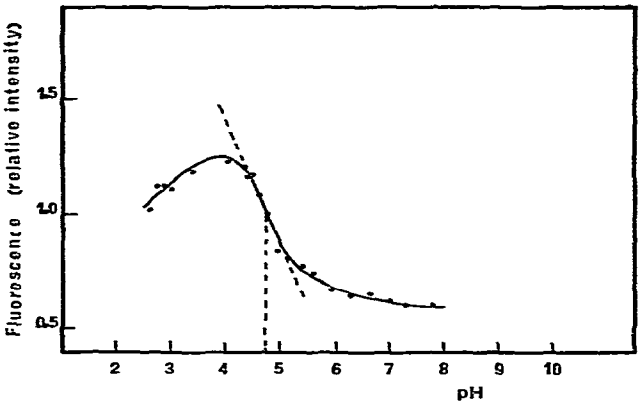

Fig. 1. Fluorescence titration of yeast 3-phosphoglycerate kinase in 0.1 M NaCl. Relative fluorescence intensity at 340 nm ($\Delta\lambda$ = 7 nm). Excitation wavelength = 295 nm ($\Delta\lambda$ = 2 nm). Protein concentration : 1 mg/ml. Temperature : 20°C. The fluorescence intensity is reported as a fraction of the value at pH 2.7.

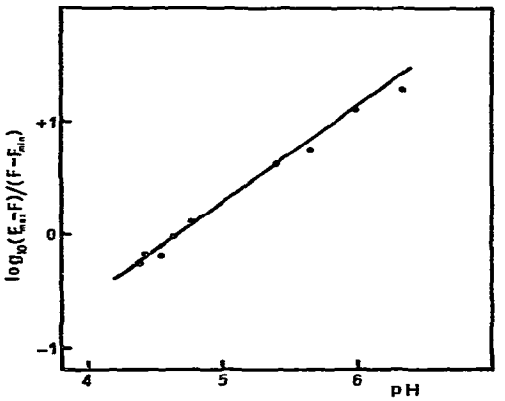

Fig. 2. Hill plot of the change in fluorescence emission as a function of pH. Ionic strength : 0.1 M NaCl. Fluorescence intensity at 340 nm ($\Delta\lambda$ = 7 nm). Excitation wavelength : 295 nm ($\Delta\lambda$ = 2 nm). Protein concentration : 1 mg/ml. Temperature : 20°C. The line drawn through the points has a slope (Hill coefficient) close to unity.

work [8] it can be seen that the relative fluorescence quantum yield of yeast 3-phosphoglycerate kinase decreases when the pH is raised from pH 3.9 to pH 7.5. pH adjustment was performed very progressively (see sect. 2). The reversibility of the phenomenon was tested and was found to be nearly complete. Some kinetic effects appeared, however, when the pH was lowered too rapidly. The curve reaches a lower plateau in the basic pH range while in the acidic one a second transition appears which does not allow the observation of the higher plateau [8].

The interpretation of the decay measurements (see below) led us to describe the fluorescence titration curve by the scheme given above in section 2.

From the extrapolation of the plot $1/(F-F_{min})$ versus $1/[H^+]$ to $1/[H^+] = 0$, one finds $F_{max}/F_{min} = 2.67$. Fig. 2 shows the plot $\log (F_{max}-F_{min})/(F-F_{min}) = f(\text{pH})$. The apparent ionization constant is $pK_a = 4.7$.

3.2. Fluorescence decay measurements

Decay measurements have been performed at two extreme pH values of the fluorescence titration curve, namely pH 3.9 (acetate buffer 5×10^{-2} M, NaCl 0.1 or 0.01 M) and pH 7.2 (Tris-HCl buffer 5×10^{-2} M, NaCl 0.1 M).

In a first series of experiments the emission wavelength was fixed at 360 nm and the transient fluorescences were measured at excitation wavelengths ranging from 260 nm to 305 nm.

In the other series of experiments the excitation wavelength was fixed at 295 nm whereas measurements were done at different emission wavelengths in the range 320–380 nm. Under these last conditions fluorescence from the protein tyrosine is negligible and the emission comes exclusively from the tryptophan residues.

As explained in the method section we separately first determined the sum of exponentials which, after convolution with the apparatus response function, individually fitted each experimental transient fluorescence. In all cases, three exponential terms were necessary to obtain a good fit. For a series of experiments, as defined above, the three decay times were found to be independent of the excitation or emission wavelength in the limits of the experimental accuracy. These results justified an analysis in which the experiments of a series were simultaneously fitted with sums of three exponentials having the same set of three decay times.

3.2.1. Variable excitation wavelength

The emission was fixed at 360 nm and the pH was 3.9. Fluorescence transients were determined at eight excitation wavelengths in the range 260–305 nm. One

Table 1
Yeast 3-phosphoglycerate kinase tryptophyl fluorescence lifetimes obtained at different excitation wavelengths (λ_{em} = 360 nm). T = 20°C, R and P are defined in sect. 2. Each transient fluorescence has been analyzed separately.

λ_{exc} (nm)	τ_1 (ns)	τ_{21} (ns)	τ_{22} (ns)	C_1	C_{21}	C_{22}	R	$P \times 10^3$
260	0.6	3.0	7.0	36.0	49.1	14.9	1.61	0.62
270	0.8	3.2	7.0	37.1	49.8	13.1	1.33	0.47
280	0.8	3.2	7.0	39.6	47.2	13.2	1.33	0.40
285	0.8	3.2	7.0	35.0	51.0	14.0	1.60	0.50
290	0.8	3.2	7.0	41.7	46.1	12.2	1.29	0.43
295	0.8	3.5	8.0	44.2	48.0	7.8	1.22	0.42
300	0.5	3.2	8.0	47.0	43.3	9.7	1.46	0.67
305	0.9	3.6	9.0	42.7	48.0	9.3	1.61	0.68

sees in table 1 the parameters obtained by analyzing separately each transient fluorescence. The simultaneous analysis of the eight experiments led to the following time constants:

$\tau_1 = 0.6$ ns, $\tau_{21} = 3.1$ ns, $\tau_{22} = 7.0$ ns.

The amplitude associated with the three time constants did not show any systematic variation with wavelength. Therefore the tryptophan fluorescence decay appeared to be independent of the excitation wavelength.

3.2.2. Variable emission wavelength

The fluorescence decays were analyzed at pH 3.9 and pH 7.2. The excitation wavelength used for this purpose was 295 nm ($\Delta\lambda_{exc}$ = 6 nm). Since tyrosine absorption is very low at this wavelength, we may assume that, under these experimental conditions, the tryptophyl fluorescence emission exclusively is observed. Six different emission wavelengths were selected. They were simultaneously analyzed. The set of three lifetimes minimizing the results was found to be the same as the one obtained in the preceding study where the emission wavelength was fixed and the excitation wavelength varied. At pH 7.2, the simultaneous analysis led to the following decay times:

$\tau_1 = 0.4$ ns, $\tau_{21} = 3.1$ ns, $\tau_{22} = 7.0$ ns.

The values of the pre-exponential terms found in these analyses at both pH are given in table 2. One can see that these parameters vary systematically with the emission wavelengths. Furthermore, in both series, the ratio $\beta = C_{21}/C_{22}$ can be considered to be independent of wavelength at the experimental accuracy.

The time constants $\tau_1, \tau_{21}, \tau_{22}$ obtained by this last analysis were then used for computing the pre-

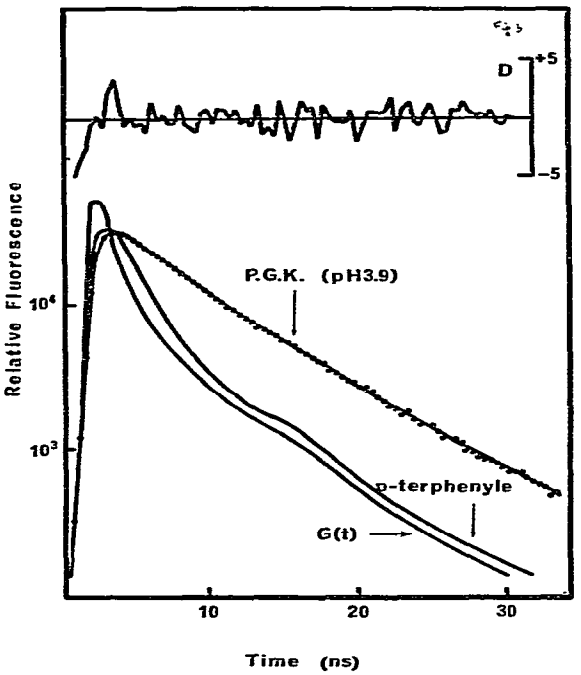

Fig. 3. Fluorescence decays of yeast 3-phosphoglycerate kinase in 0.05 M acetate buffer pH 3.9, 0.1 M NaCl. Excitation wavelength : 295 nm, $\Delta\lambda$ = 6 nm. Emission wavelength : 360 nm, $\Delta\lambda$ = 12 nm. Temperature : 20°C. The solid lines are calculated convolution products. The points are related to experimental values. The insert is a representation of the deviation between convolution products and experimental values (see Materials and Methods).

Table 2
Yeast 3-phosphoglycerate kinase tryptophyl fluorescence lifetimes obtained at different emission wavelengths (λ_{exc} = 295 nm). T: 20°C. R and P are defined in sect. 2. The transient fluorescences measured at a given pH were analyzed simultaneously by assuming they were all characterized by the same decay times.

	pH 3.9			pH 7.2		
	τ_1 = 0.6	τ_{21} = 3.1	τ_{22} = 7.0	τ_1 = 0.4	τ_{21} = 3.1	τ_{22} = 7.0
λ_{em} (nm)	C_1	C_{21}	C_{22}	C_1	C_{21}	C_{22}
320	66.4	26.9	6.70	93.8	5.0	1.2
330	56.7	34.6	8.7	92.4	6.1	1.5
340	45.4	43.7	10.9	90.4	7.7	1.9
350	37.6	49.9	12.5	90.7	7.4	1.9
360	31.7	54.7	13.6	88.5	9.2	2.3
380	19.1	64.7	16.2	85.5	11.6	2.9

exponential terms C_1, C_{21}, C_{22} for different values of β as described in the method section. Fig. 4 shows the variation of the parameter ΣP_i with β for each series of experiments. It can be seen that the value $\beta = 4.0$ is consistant with all experiments. The average lifetime of tryptophan 2 is then:

$$\langle \tau_2 \rangle = \frac{\beta\tau_{21} + \tau_{22}}{1 + \beta} = 3.9 \text{ ns}$$

The constant ratio β along the excitation and the emission spectrum allows to rationalize our results as follows. The shorter lifetime τ_1 is ascribed to the tryptophan residue number 1, while the two lifetimes τ_{21} and τ_{22} correspond to two types of environment of the second tryptophan residue of the protein. The results are thus consistant with two fluorescent species. The simultaneous analysis of a series of experiments with the same decay times increases the accu-

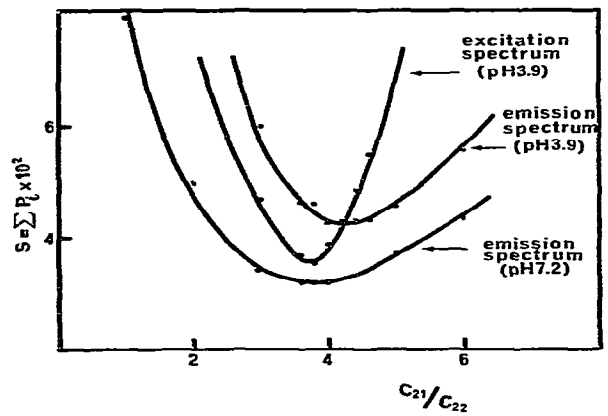

Fig. 4. Variation of the sum $S = \Sigma_i P_i$ with the ratio $\beta = C_{21}/C_{22}$ for three sets of experiments.

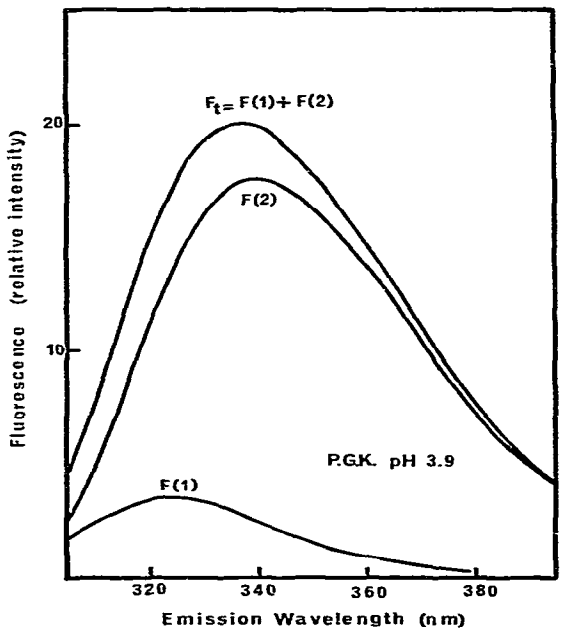

Fig. 5. Resolution of the fluorescence spectrum of yeast 3-phosphoglycerate kinase at pH = 3.9 (acetate buffer 5 × 10^{-2} M, NaCl 0.1 M). Component 1 is characterized by τ_1 = 0.6 ns, component 2 by τ_{21} = 3.1 and τ_{22} = 7.0 ns (C_{21}/C_{22} = 4.0).

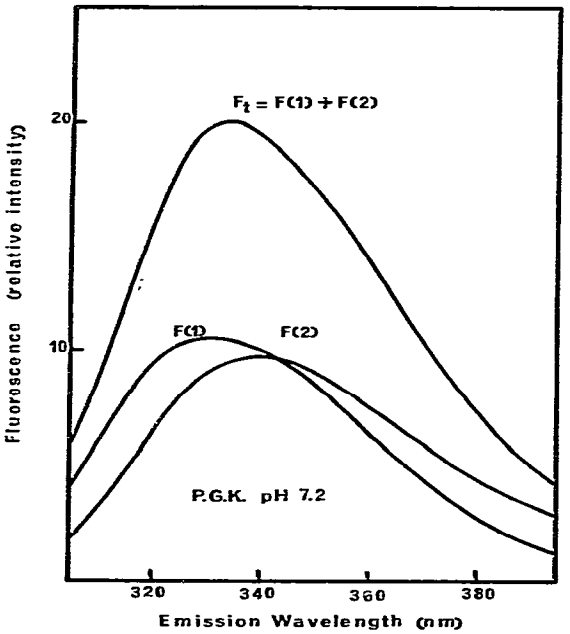

Fig. 6. Resolution of the fluorescence spectrum of yeast 3-phosphoglycerate kinase at pH = 7.2 (Tris-HCl buffer 5 × 10^{-2} M, NaCl 0.1 M). Component 1 is characterized by τ_1 = 0.4 ns, component 2 by τ_{21} = 3.1 and τ_{22} = 7.0 ns (C_{21}/C_{22} = 4.0).

racy on the decay parameters. This has been recently discussed in several works [15,18,19].

According to the analysis described in section 2, we have determined the two resolved fluorescence spectra arising from each tryptophan residue. The results are illustrated by figs. 5 and 6. When the areas under each of the resolved spectra are compared, it appears that component 2 contributes 87% of the total emission at pH 3.9, while the two components contribute almost equally to the spectrum at pH 7.2. The smaller quantum yield at pH 7.2 observed by steady state fluorescence must be attributed then to a static quenching of tryptophan residue number 2.

3.2.3. Determination of the intrinsic dissociation and the isomerization constants

Following our interpretation of the fluorescence decays one can write for state I:

$$\langle\tau_I\rangle = \tau_1 .$$

According to relations (8) and (9), the ratio of the maximum to the minimum of the fluorescence titration curve is then given by:

$$\frac{F_{max}}{F_{min}} = \frac{1+K_2}{1+(\langle\tau_I\rangle/\langle\tau_{II}\rangle)K_2}, \tag{16}$$

where $\langle\tau_{II}\rangle = 0.5\ \tau_1 + 0.5\ \langle\tau_2\rangle = 2.2$ ns and $\langle\tau_2\rangle$ is the average decay time of tryptophan number 2.

K_2 can be obtained by resolving the eq. (16) where F_{max}/F_{min} τ_1 and $\langle\tau_{II}\rangle$ are replaced by their experimental values. With $\tau_1 = 0.5$, one finds:

$$K_2 = 4.28. \tag{17}$$

Then, using relation (5), one obtains $pK_1 = 5.42$. At pH 7.2, the concentration $[A_1]$ and $[A_2]$ of tryptophan 1 and 2 are:

$$[A_1] = [P^-] + [P{-}TR^-], \qquad [A_2] = [P^-],$$

from which one obtains:

$$[A_1]/[A_2] = 1+K_2. \tag{18}$$

If one applies eq. (15) to the fluorescence decay obtained at pH 7.2, one finds:

$$[A_1]/[A_2] = 7.8,$$

which leads to $K_2 = 6.8$. (19)

This value is somewhat higher than the value obtained by the first determination. But it must be noticed that the accuracy of both determinations is not high since the value of τ_1 can be affected by an important error. K_2 known, the values of $[A_2]/[A_1]$ can be obtained at all pH values since

$$[A_1] = [P_0], \qquad [A_2] = [PH] + [P^-].$$

Taking into account eqs. (4) and (5) one can write:

$$[A_2]/[A_1] = \frac{1}{1+\alpha} \times \left[1 + \frac{\alpha}{1+K_2}\right], \tag{20}$$

where $\alpha = K_a/[H^+]$.

One can easily see that at pH 3.9, eq. (20) can be reduced to:

$$[A_2]/[A_1] = 1/(1+\alpha) = 0.86.$$

On the other hand, by applying at pH 3.9 the relation (15) to the component spectra obtained, one finds:

$$[A_2]/[A_1] = 1.03.$$

Taking into account the many sources of inaccuracy, these two values can be considered as being in fair agreement with each other.

4. Discussion

At both pH values studied and at all excitation and emission wavelengths chosen, the fluorescence decay of yeast 3-phosphoglycerate kinase was found to be the sum of three exponential functions.

When the emission wavelength was fixed at 360 nm, the decay was independent of the excitation wavelength. Under these conditions one expects to observe exclusively the tryptophyl fluorescence. These results show that the emission does not contain any contribution from the tyrosine fluorescence, neither from Rayleigh nor Raman scattering. Otherwise the decay would depend on the excitation wavelength. Consequently, despite its low value, the decay time τ_1 must be attributed to the tryptophyl emission. A very short lifetime has also been recently reported by Torikata et al. [18] for the tryptophan fluorescence of Pig heart lactate dehydrogenase.

By contrast, the decay time of the fluorescence excited at 295 nm depended on the emission wavelength. By a quantitative analysis of this dependence, we were able to decompose the spectrum of yeast 3-phosphoglycerate kinase into two distinct spectra which were attributed to the two tryptophyl residues of the protein molecule. These spectra are shifted by 6 nm with respect to each other. The blue one (tryptophan residue number 1) corresponds to the lifetime $\tau_1 = 0.4$—0.6 ns. τ_1 characterizes a state strongly quenched of tryptophan 1 in the protein. We do not know the quenching mechanism giving rise to τ_1. But from its efficiency and from the maximum of its resolved spectrum, we can predict that tryptophan 1 is probably buried in the protein core.

Both lifetimes $\tau_{21} = 3.1$ ns and $\tau_{22} = 7.0$ ns have been attributed to tryptophan 2. The occurrence of a multiexponential decay has already been described for cyclopeptides and proteins containing a single tryptophan residue [2,20]. In contrast, for simple indole derivatives dissolved in different water-organic solvent binary mixtures, we found a continuous evolution of single lifetimes from pure water to pure organic solvent [21]. It can be concluded from these previous observations that the multiexponential decay of a tryptophan residue in a protein has to be related to several configurations of the local environment of that residue. This applies to tryptophan 2 of yeast 3-phosphoglycerate kinase.

τ_{22} seems to arise from a configuration where perturbations by vicinal groups of the protein are relatively small. In contrast, the value τ_{21} which corresponds to the second state of tryptophan 2, results from dynamic interactions with a vicinal quencher group. The ratio C_{21}/C_{22}, which does not depend on the emission wavelength and pH, represents the relative populations of the two distinct states. From the maximum of its resolved fluorescence spectrum, it can be concluded that tryptophan 2 is probably located a more hydrophilic domain than tryptophan 1 was. In addition, it is worthy to note that a long time constant also appeared in the decays of HSA [20], of WGA [22] and the lac repressor protein of E. coli [3], three proteins for which the fluorescence emission arises from highly solvated emitters.

The variation of the fluorescence intensity between pH 3.9 and 7.2 may be fitted with a Henderson-Hasselbach titration curve having a $pK_a = 4.7$. These results suggest that the fluorescence of tryptophan 2 is quenched by a carboxylate anion. According to the decay results at pH 7.2, the quenching occurs by formation of a complex in the ground state between the carboxylate and the indole ring. One may propose that this complex is stabilized by a hydrogen bond between the indolic N—H group and the carboxylate group. This implies that an aspartic or a glutamic acid residue is situated in the vicinity of the tryptophan residue. In its acid form such a group might induce a dynamic quenching of the indole ring by a charge transfer process [23]. This mechanism might explain the presence of a short and a long time constant in the fluorescence decay of tryptophan number 2 if the contact between the carboxyl and the indole ring is hindered by a rotation barrier (2).

The interaction of the indole ring with the negatively charged group is an isomerization reaction the constant of which was designated by K_2. There are two ways of computing K_2, which lead to similar values. The determination of the concentration ratio of the two kinds of emitting tryptophan residues at pH 3.9 shows that the time spectrum analysis is consistent with the fluorescence titration curve. This result shows that energy transfers between tryptophyl residues are small, otherwise relation (15) would not be valid. This point has been discussed in a preceding work in the case of the lac repressor protein [3] and will be studied further in the case of P.G.K. in a future work.

Let us note that the time spectrum analysis is based on the relation $k_{F_1}\epsilon_1 = k_{F_2}\epsilon_2$. As discussed before, the relation $\epsilon_1 = \epsilon_2$ comes from the analysis of the fluorescence decay at different wavelengths of excitation. Secondly, in a recent work [21] we showed that for solutions of indole compounds, k_F varies linearly with the wavenumber ν_F of the fluorescence maximum spectrum. In contradistinction, the variation of k_F with ν_F was shown to be very small in the case of tryptophyl residues of proteins. Since the shift between the two resolved spectra which characterize both residues is small, one can reasonably consider that $k_{F_1} = k_{F_2}$.

In conclusion, the decomposition of the tryptophyl fluorescence of yeast 3-phosphoglycerate kinase into two resolved spectra characterizing each tryptophan residue appears to be satisfactory. This implies that tryptophan energy transfers between these residues are small. Our analysis allows us to conclude that the fluorescence titration observed in the pH range 3.9–8.0, affects only one of the residues, probably through a ground state interaction with a carboxylate anion.

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