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PULSE FLUORIMETRY STUDY OF ENERGY TRANSFERS BETWEEN TRYPTOPHAN RESIDUES AND NADPH IN BEEF LIVER GLUTAMATE DEHYDROGENASE COMPLEXES

J. C. BROCHON[a], Ph. WAHL[a], J. M. JALLON[b] and M. IWATSUBO[b]

[a]*Centre de Biophysique Moléculaire, C.N.R.S., 45045 Orleans Cédex and* [b]*Centre de Génétique Moléculaire, C.N.R.S., 91190 Gif-sur-Yvette (France)*

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SUMMARY

A method is proposed to determine the rates of singlet energy transfers in an array of chromophores containing a finite number of donors and fluorescent acceptors. This method is based on measurements of transfer efficiency coupled with pulse fluorimetry. Three classes of donors can be distinguished which differ in their energy transfer rate. The rates of the first, the second and the third class are respectively greater than, of the order of, and smaller than the emission rate. The method is applied to the study of the energy transfers from tryptophan residues to NADPH, in ternary and quaternary glutamate dehydrogenase complexes. Practically, all these tryptophan residues belong to the first class. They can be divided into two subclasses having different transfer rate values. The distances between these residues and the NADPH site are of the order of 2.5 nm. In addition, the ligand binding induces a protein conformational change, leading to a fluorescence quenching of the tryptophanyl emission.

INTRODUCTION

Measurements of excitation transfer efficiency between chromophores in the singlet state have often been made in order to determine the distance between different parts of biological macromolecules or macromolecular associations [1].

It has been pointed out that measurements of fluorescence decays of the donors would strengthen the potentiality of the method [2]. Theoretical and experimental studies have been performed in systems in which flexible macromolecules each contained one donor and one acceptor chromophore separated by a distance which is distributed according to a continuous law [3, 4].

However, there are a number of biological systems where a quite different situation occurs. This is the case of complexes made of an enzyme and a fluorescent

Abbreviations: NADPH, reduced nicotinamide adenine dinucleotide phosphate; NADH, reduced nicotinamide adenine dinucleotide; GTP, guanosine triphosphate.

coenzyme, as the NADH or NADPH-containing dehydrogenases [5–8]. It has been shown that in these complexes, excitation transfers occur from the enzyme tryptophans towards the coenzyme bound to the protein. Generally speaking, one expects here a rather complicated situation. If the enzyme contains several tryptophan residues, the intrinsic emission properties would generally differ for each residue. Furthermore the distance and the mutual orientations between the tryptophan residues and one given NADH or NADPH ligand, are generally different, which entails a difference in the rates of transfers. Additional complications occur when the enzyme molecule binds several NADH or NADPH ligands.

It can be shown that, under given conditions, the rate of energy transfers occurring between the donor-acceptor couples of such a chromophore array, can be obtained by measuring in the same complexes the global transfer efficiency on the one hand and on the other hand, the transient fluorescence obtained by choosing the wavelengths of excitation and emission according to the following conditions: (1) direct excitation and emission of donors; (2) direct excitation and emission of acceptors; (3) excitation of donors, emission of acceptors (Wahl, Ph., unpublished).

The principle of the method is given in this work in a form appropriated to the study of two glutamate dehydrogenase-NADPH complexes. The donors here are the enzyme tryptophans, while the acceptor is the coenzyme bound to the enzyme. The measurements of the transfer efficiency and of the transient fluorescence corresponding to the third condition, are described later.

The transient fluorescence obtained under the two first conditions was measured in a preceding work [9].

The complicated structure of the system under study does not allow a detailed description of all the transfer process to be given. However, the analysis of the results led us to distinguish two kinds of tryptophan residue differing in their excited state lifetime and their rate of energy transfers to NADPH.

MATERIALS AND METHODS

(*a*) *General*

Experimental details about the compounds and the photocounting pulse fluorimeter have been given in our preceding work [9].

The excitation light was provided by an hydrogen flash lamp, followed by a Bausch and Lomb 500 nm monochromator, which selected a wavelength band centered at 295 nm, with a 10 nm width. The emission light was filtered through a high intensity Bausch and Lomb monochromator set up at 460 nm with a band width of 32 nm. The P.M. was a XP 2020 Radiotechnique.

The fluorescence decays were assumed to be a sum of exponential functions which could be written:

$$I(t) = \sum_{i=1}^{n} B_i \exp(-t/\tau_i) \tag{1}$$

where the parameters B_i and τ_i were obtained by analyzing the experimental transient fluorescence, as described in our previous work [9]. 1,1,4,4-Tetraphenyl-1,3-butadiene dissolved in deaerated cyclohexane, was used as a reference solution for that analysis.

(*b*) *Transient fluorescence analysis in the case of energy transfers*

It could be considered that the exciting light with a wavelength of 295 nm was mostly absorbed by tryptophan residues of the glutamate dehydrogenase-NADPH complexes, and that only a small fraction of it was absorbed by bound NADPH, since at that wavelength the molar absorbtion of that coenzyme is small [1]. A part of the exciting energy was re-emitted into two distinct fluorescent bands: the tryptophan residue emission centered at 350 nm, and that of NADPH centered at 460 nm [5–8]. On the other hand, the exciting light with a wavelength of 336 nm was absorbed only by the bound NADPH molecules and re-emitted in the NADPH fluorescent band.

In order to analyse the transient fluorescence emitted in these two bands, the following simplifying assumptions were made: all the NADPH sites could be considered as identical. Each tryptophan residue transfered its energy to one bound NADPH molecule only.

However, it was expected that the environment of each of the tryptophan residues and its distance from the nicotinamide site was specific. Three classes of tryptophan residues have to be envisaged with regard to the rate at which they transfer their energy to NADPH.

The first class is constituted by residues having a high rate of energy transfers. They do not contribute to the 350 nm emission. Their contribution to the 460 nm transient emission is similar to the emission obtained with direct NADPH excitation. Their distance from NADPH is smaller than the critical transfer distance.

The second class concerns residues, the rate of transfer of which is comparable to their rate of emission. These tryptophans contribute both to emissions at 350 nm and at 460 nm. This 460 nm transient emission was expected to be lengthened by the transfer delays, in contrast with the direct emission of NADPH excited at 336 nm. The distance of one of these tryptophans from NADPH is also expected to be of the order of the critical transfer distance.

The third class of tryptophan residues do not transfer their energy to NADPH. They only contribute to the 350 nm emission. They are separated from NADPH by a distance greater than the critical transfer distance.

Under these conditions, the decay of the tryptophyl fluorescence excited at 295 nm, and emitted at 350 nm can be written as follows:

$$I_d(t) = \sum_{i=1}^{n} C_{2i} \exp(-k'_{2i}t) + \sum_{j=1}^{m} C_{3j} \exp(-k_{3j}t) \tag{2}$$

Where 2 and 3 designate the tryptophan classes defined above, i and j represent subclass indexes. These subclasses are characterized by their rate of deactivation k_{3j} and k'_{2i} where

$$k'_{2i} = k_{2i} + \mu_{2i} \tag{3}$$

and μ_{2i} the rate of energy transfer to NADPH, k_{2i} the deactivation rate, which would be observed if the transfer was inefficient.

$$C_{2i} = A_{2i}/A_F, \; C_{3j} = A_{3j}/A_F \tag{4}$$

Where A_{2i} and A_{3j} are the contribution to the absorbance at 295 nm of the subclasses $2i$ and $3j$, and A_F the sum of the absorbance of the fluorescent tryptophans:

$$A_F = \sum_{i=1}^{n} A_{2i} + \sum_{j=1}^{m} A_{3j} \tag{5}$$

n and m are the numbers of tryptophan subclasses in each class. Expression 4 is valid if the radiative deactivation rates are identical for all the tryptophan residues. This condition can be considered as approximately realised. We also consider the total absorbance defined by Eqn. 6:

$$A = A_F + A_1 \tag{6}$$

Where A_1 is the sum of the absorbances at 295 nm of the tryptophans which transfer with high rates, (and did not contribute to the 350 nm fluorescence), and of the absorbance of NADPH at that wavelength.
We defined:

$$C_1 = A_1/A_F \tag{7}$$

From Eqns. 6 and 7 one obtains:

$$\frac{A_F}{A} = \frac{1}{1+C_1} \tag{8}$$

For our purpose we assume that the fluorescence decay of directly excited NADPH can be approximated by a mono-exponential function as follows:

$$I_a(t) = I_a(0) \exp(-k_a t) \tag{9}$$

Then the transient fluorescence emitted at 460 nm and excited at 295 nm can be written [2, 12]:

$$I_{aT} = K\left\{\sum_{i=1}^{n} \frac{C_{2i}\mu_{2i}}{k_a - k_{2i}} (\exp(-k'_{2i}t) - \exp(-k_a t)) + C_1 \exp(-k_a t)\right\} \tag{10}$$

Where K is a constant, the determination of which will be given below. Expression 10 can be rewritten as:

$$I_{aT} = \sum_{i=1}^{n} B_{2i} \exp(-k'_{2i}t) + B_a \exp(-k_a t) \tag{11}$$

where

$$B_{2i} = \frac{KC_{2i}\mu_{2i}}{k_a - k'_{2i}} \tag{12}$$

and

$$B_1 = \sum_{i=1}^{n} B_{2i} + B_a = KC_1 \tag{13}$$

B_1 and C_1 are equal to zero, whenever the absorption of the acceptor and of the donors transferring at high rate are negligible.

Transfer efficiency. The fluorescence quantum yields of the acceptor directly excited, q_A, and indirectly excited through the donor, q'_A, were linked by the relation:

$$\eta = \frac{q'_A}{q_A} \tag{14}$$

where η was the transfer efficiency of the donor. Under our experimental conditions, only a fraction, γ, of the coenzyme binding sites was occupied. The overall transfer efficiency from tryptophans to coenzyme was then:

$$\eta = \eta_{app}/\gamma \tag{15}$$

where

$$\eta_{app} = \frac{F_{295}}{F_{335}} \times \frac{L_{335}}{L_{295}} \times \frac{T_{335}}{T_{295}} \times \frac{A_{(335)}}{A_{(295)}} \tag{16}$$

The indices 295 and 335 correspond to the excitation wavelengths.

F_{295} and F_{335} are the fluorescence intensities emitted at 460 nm by the studied sample. These intensities were measured with a Jobin-Yvon spectrofluorimeter using wavelength bands of 2, 3 and 7 nm at 295, 335 and 460 nm, respectively.

L_{295} and L_{335}, were the intensities of the exciting lamp. The fluorescence intensities of a dilute solution of quinine sulfate in 0.05 M H_2SO_4 ($A = 0.02$ at 350 nm) were measured, using the 295 nm and 335 nm excitation. The ratio of these intensities multiplied by the inverse of the absorbances of the solution at these wavelengths provided the value of L_{295}/L_{335}. The same slit width was used in the spectrofluorimeter for the sample studied and the quinine sulfate solution. $A_{(295)}$ and $A_{(335)}$ were the absorbances of the solution; T_{295} and T_{340} were the transmissions of the solution which affected the inner filter effect, and which, for the apparatus used is given by the expression:

$$T_\lambda = 10^{-A(\lambda)/2}$$

With the assumption already made, the transfer efficiency is given by expression 17:

$$\eta = \frac{A_F}{A}\left(\sum_{i=1}^{n} C_{2i}\eta_{2i} + C_1\right) \tag{17}$$

and

$$\eta_{2i} = \frac{\mu_{2i}}{k'_{2i}} \tag{18}$$

Determination of the transfer rates. Taking into account relations 18, 12, 13 and 17, the constant K can be expressed by Eqn. 19:

$$K = \frac{1}{\eta} \cdot \frac{A_F}{A}\left(\sum_{i=1}^{n} \frac{B_{2i}(k_a - k'_{2i})}{k'_{2i}} + B_1\right) \tag{19}$$

The set of Eqns. 7, 13 and 19 can be resolved by successive approximations and provide the values of the unknown K, A_F/A and C_1. The other parameters entering in these equations are obtained by the transient fluorescence analysis and the transfer efficiency determination. Then μ_{2i} and η_{2i} are found by using expressions 12 and 18.

We then consider all the chromophores absorbing at 295 nm. The fractions of the different classes and subclasses are obtained by:

$$f_{2i} = C_{2i}\frac{A_F}{A},\ f_{3j} = C_{3j}\frac{A_F}{A},\ f_1 = C_1\frac{A_F}{A} \tag{20}$$

Estimation of the energy transfer distances. The rate of energy transfer μ_{2i} can be written [1]:

$$\mu_{2i} = k_{2i}\left(\frac{R_{2i}^{(0)}}{R_{2i}}\right)^6 \tag{21}$$

where R_{2i} is the actual distance between the donor and acceptor molecule, $R_{2i}^{(0)}$ the critical transfer distance which is defined by the classical relation [1]:

$$R_{2i}^{(0)} = 8.8 \cdot 10^{-25}\Phi_{2i}K_{2i}^2 v^{-4} J_{2i} \tag{22}$$

where Φ_{2i} is the quantum yield of the chromophores of the subclass $2i$, J_{2i} an integral which depends on the overlap of the donor absorption spectrum and the acceptor emission spectrum, v the medium refractive index, K_{2i} an angular factor.
If in Eqn. 21, $R_{2i}^{(0)}$ is replaced by the right side of expression 22 μ_{2i} appears to be proportional to the product $k_{2i}\Phi_{2i}$ which is equal to the radiative rate of deactivation k_{D2i}. A crude estimation of R_{2i} can be obtained by adopting the following simplifying assumptions: k_{D2i} will be taken equal to the deactivation radiative rate of free tryptophan in aqueous solutions. So one has $k_{D2i} = k\Phi$ where k is the total rate of deactivation ($k = 3.3 \cdot 10^8\ s^{-1}$ according to ref. 10) and Φ the quantum yield of an aqueous tryptophan solution; v is equal to 1.33, the water refractive index, K^2 has an average value of 2/3 [1]. With these assumptions Eqn. 21 can be written:

$$\mu_{2i} = k\left(\frac{R_0}{R_{2i}}\right)^6 \tag{23}$$

where R_0 is the critical distance corresponding to the free tryptophan-free NADH aqueous solutions. R_0 has been determined previously [1] ($R_0 = 2.5$ nm).

RESULTS AND DISCUSSION

Two enzyme complexes have been studied in this work: the ternary complex glutamate dehydrogenase-NADPH-L-glutamate and the quaternary complex glutamata dehydrogenase-NADPH-L-glutamate-GTP. For the measurements of the transient 460 nm fluorescence obtained with a 295 nm excitation the concentrations of glutamate dehydrogenase protomers, NADPH, L-glutamate and GTP were, respectively, 70 μM, 28.5 μM, 45 mM and 450 μM. For the transfer efficiency measurements, these concentrations were very similar namely 70 μM, 28 μM, 45 mM and 470 μM.

The transient fluorescence emitted at 460 nm and excited at 295 nm showed a quite different shape from the transient fluorescence emitted at the same wavelength, but excited at 336 nm. This last curve had been measured in our preceding work [9] (see Fig. 1). The difference could be easily characterized by examining the distance between the barycenters of the transient fluorescence and the apparatus response

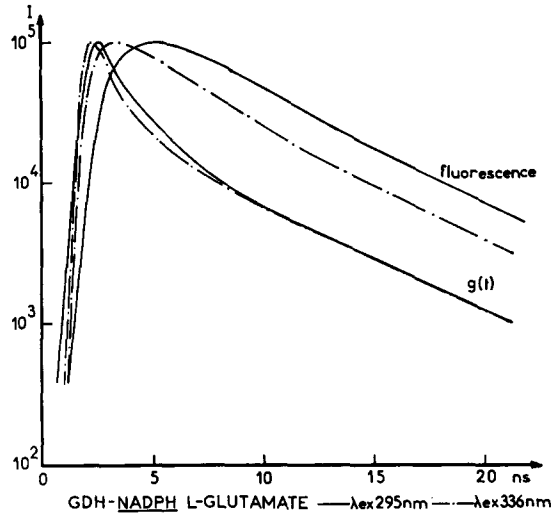

Fig. 1. Apparatus response $g(t)$ of the pulse fluorimeter and transient fluorescence of the glutamate dehydrogenase-NADPH-L-glutamate complex emitted at 460 nm with excitation wavelength at 336 nm (· —·) and 295 nm (—).

function. This distance was greater with the 295 nm than with the 336 nm excitation.

It has been shown in our previous work that the fluorescence decay of glutamate dehydrogenase-NADPH complexes, using the 336 nm excitation, were sums of two exponentials with positive amplitudes [9]. This was not the case with the 295 nm excitation: neither a single exponential decay nor a sum of two or three exponentials with positive amplitudes could fit the data. We tried to interprete these data by using the theoretical expression (Eqn. 11). The values of k_a were taken equal to $1/\langle\tau_a\rangle$ where the values of $\langle\tau_a\rangle$ were the average decay time of NADPH fluorescence measured in our previous work for each complex. In addition the value of n had to be determined. A good fit could be obtained with $n = 2$; while this was not possible with $n = 1$.

The result of such an analysis is shown on Fig. 2. The values of the parameters obtained are given in Table I. It can be seen in this table that B_1 was different from zero, for both complexes studied. As it will be discussed below, this result can probably be explained by the small absorption contribution of NADPH at 295 nm.

According to the theoretical expressions given above, the time constants

$$\tau'_{21} = \frac{1}{k'_{21}} \text{ and } \tau'_{22} = \frac{1}{k'_{22}}$$

represent the decay times of two subclasses of tryptophan residues of class 2. They should also appear in the decays of the tryptophan fluorescence emitted at 350 nm. The corresponding transient fluorescences had been measured in our previous work and analysed by assuming two exponential decays. The decay times obtained by that analysis were 1.5 ns and 5.2 ns in the case of the glutamate dehydrogenase-NADPH-glutamate complex, 1.36 ns and 4.6 ns in the case of the quaternary complex (Table 5 of ref. 9). These values are different from τ'_{21} and τ'_{22} measured in the present work. We then tried to fit three exponential decays to the transient tryptophyl fluores-

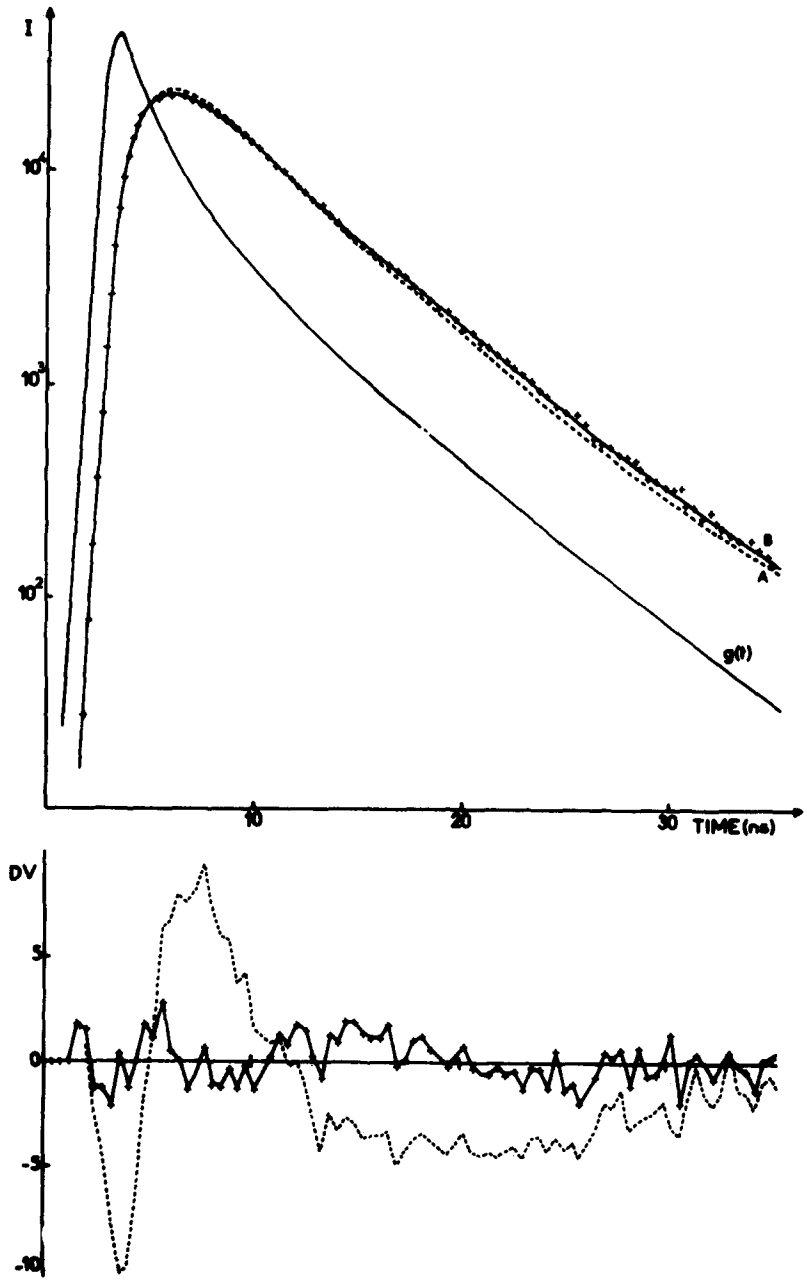

Fig. 2. Fluorescence of glutamate dehydrogenase-NADPH-L-glutamate complex excited at 295 nm and emitted at 460 nm. Upper curves: Apparatus function $g(t)$, experimental transient emission (+ + +), calculated product of convolutions of $g(t)$ by the time-dependent intensity given by formula 10 with the best value of the parameters the number of donor subclasses n being 1 (- - - -) or 2 (—). Lower curves: Deviation functions $DV = I_{calc} - I_{exp}/\sqrt{I_{exp}}$ where I_{exp} the experimental transient fluorescence and I_{calc} the best convolution drawn above with $n = 1$ (- - - - -) or $n = 2$ ($\times - \times$).

TABLE I

PARAMETERS RESULTING FROM THE ANALYSIS OF THE TRANSIENT FLUORESCENCE OF GLUTAMATE DEHYDROGENASE-NADPH COMPLEXES OBTAINED WITH AN EXCITATION AND EMISSION WAVELENGTH OF 295 nm AND 460 nm, RESPECTIVELY

The residual Res is defined by

$$\text{Res} = \frac{1}{N}\sum_{j=1}^{N}\frac{(I_{exp}{}^{j}-I_{calc}{}^{j})^2}{I_{exp}{}^{j}}$$

where $I_{exp}{}^{j}$ and $I_{calc}{}^{j}$ are the counts in the experimental transient fluorescence and in the calculated convolution at channel j. N is the total number of channels. Other parameters are defined in the text.

GDH+NADPH	τ'_{21} (ns)	τ'_{22} (ns)	τ_a (ns)	A_{21}	A_{22}	A_a	Res
+L-Glu	0.85	3.4	1.84	−0.098	0.044	0.064	1.26
+L-Glu+GTP	0.85	3.65	1.55	−0.174	0.035	0.166	2.09

TABLE II

PARAMETERS RESULTING FROM THE ANALYSIS WITH THREE EXPONENTIAL DECAYS OF THE TRANSIENT FLUORESCENCE OF GLUTAMATE DEHYDROGENASE-NADPH COMPLEXES WITH AN EXCITATION WAVELENGTH OF 295 nm AND AN EMISSION WAVELENGTH OF 350 nm.

The decay times τ'_{21} and τ'_{22} are taken from Table I. The other parameters are determined by the analysis. GDH, glutamate dehydrogenase.

GDH+NADPH	τ'_{21} (ns)	τ'_{22} (ns)	τ_3 (ns)	C_{21}	C_{22}	C_3	Res
+L-Glu	0.85	3.4	7.6	0.56	0.38	0.06	1.33
+L-Glu+GTP	0.85	3.65	8.5	0.67	0.315	0.11	1.43

TABLE III

PARAMETERS CHARACTERIZING THE TRYPTOPHYL EMISSIONS AND THE ENERGY TRANSFERS TOWARDS NADPH IN GLUTAMATE DEHYDROGENASE COMPLEXES

The meanings of the symbols are given in the text. GDH, glutamate dehydrogenase.

GDH+NADPH	μ_{21} (ns^{-1})	μ_{22} (ns^{-1})	R_{21} (nm)	R_{22} (nm)	τ_{21} (ns)	τ_{22} (ns)	f_{21}	f_{22}	f_3	f_1
+L-Glu	0.465	0.125	2.36	2.94	1.41	6.21	0.54	0.37	0.06	0.04
+L-Glu+GTP	0.396	0.119	2.42	2.96	1.28	6.45	0.63	0.29	0.01	0.07

cence, in which two of the decay times were taken equal to τ'_{21} and τ'_{22} shown in Table I of the present work. We could then obtain good fits and the parameters obtained were brought in Table II. The presence of a third component in the tryptophan decay means that there is a fraction of these residues which do not transfer their energy to NADPH. However, this fraction is small, as the values of C_3 brought in Table III, showed.

Transfer efficiency determination. Under the conditions of concentration used for the efficiency measurements, the fraction of saturation was 40 %. With the method described above, we obtained a value $\eta = 0.41$ for both complexes.

Calculation of the transfer parameters. By using the method described at the beginning of this work, it was possible to determine the following parameters characterizing the two subclasses of tryptophans of class 2: the transfer rates μ_{21} and μ_{22}, the decay times

$$\tau_{21} = \frac{1}{k_{21}} \text{ and } \tau_{22} = \frac{1}{k_{22}}$$

which would be observed in the absence of transfers, the transfer efficiencies η_{21} and η_{22} the fractions f_{21} and f_{22} of these tryptophans and the fraction f_3, of non-transferring tryptophans. Finally, by applying the relation 23 the distance R_{21} and R_{22} between the tryptophans and the NADPH site could be obtained. These parameters are gathered in Table III.

According to this table, the results obtained with the ternary and the quaternary complexes look very similar. The fraction f_3 of the non-transferring tryptophans is very small. This can be explained by a small amount of the enzyme which does not bind NADPH. The fractions f_1 of chromophores emitting at 460 nm without transfer delay are also very small and can be attributed to the absorption of bound NADPH at 295 nm. The values of f_1 are indeed comparable to the contribution which can be calculated it one admitted that the absorptivity values of bound and free NADPH are identical at 295 nm.

A protomer of glutamate dehydrogenase contains four tryptophan residues. The decay analysis does not allow determination of the fluorescence decay times of each residue. Our analysis, however, takes into account the emission heterogeneity since we can distinguish two main kinds of tryptophan residues, the decay times of which are τ'_{21} and τ'_{22}, respectively. These decay times are very different and correspond to different quantum yield values. We showed that these residues belong to class 2, which means that the rate of energy transfers to the bound coenzyme is comparable to the deactivation rate. According to expression 21, the distances between these residues and the coenzyme are close to the critical transfer distance, which is given by expression 22. The overlap integral J and the quantum yield Φ values we used are based on the measurements performed by others on the aqueous solutions of tryptophan and the coenzyme. These evaluations should be improved in future works by measuring accurately the spectroscopic properties of the enzyme complexes. K^2 depends on the mutual orientations of the donor and acceptor electronic transition moments, which are not known. In our calculations, we adopted the value $K^2 = 2/3$, which is the average value of K^2 in an isotropic solution. This choice is evidently arbitrary and entails an uncertainty on the calculated distance.

No tryptophan appears to be in close proximity to NADPH in the complexes.

It is also found that the long decay times τ_{22} of the tryptophan fluorescence are practically equal to the decay time τ_2 of the enzyme in the absence of ligands (compare Table III of the present work with Table V of ref. 9). The small decay times τ_{21}, however, are smaller and their contributions are greater than the decay time τ_1 of the non-complexed enzyme. This result means that the ligands induce a change in the close environment of some of the enzyme tryptophan residues, which decreases the lifetime and therefore the quantum yield of these chromophores. This effect, added to the energy transfers, contributed to the overall quenching of the enzyme tryptophyl fluorescence. From the mechanism of the reaction catalysed by glutamate dehydrogenase, it is expected that the glutamate and NADPH sites are close to each other.

According to our estimation of distances between the tryptophan residues and the coenzyme, we should conclude that direct interactions between the ligands and the tryptophan residues are not probable. Then the tryptophyl environment changes induced by ligand binding might be brought about by a non-local conformational change of the protein. The similarity between the ternary and the quaternary complexes leads to similar conclusions about this last complex.

In conclusion, we presented in this work a new approach of the study of excitation transfers between tryptophan residues and the coenzyme NADPH in glutamate dehydrogenase complexes. Pulse fluorimetry has allowed us to distinguish several tryptophan residue classes and subclasses, differing by their quantum yield and their rate of excitation transfers to the coenzyme. This method can be generalised to other biological systems and especially to the other NADP(H) dehydrogenase complexes.

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