Pulse Fluorimetry Study of Beef Liver Glutamate Dehydrogenase–Reduced Nicotinamide Adenine Dinucleotide Phosphate Complexes†

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ABSTRACT: Single photon counting pulse fluorimetry has been used in order to study the two ternary complexes GDH-GTP-NADPH and GDH-L-glutamate-NADPH and the quaternary complex GDH-GTP-L-glutamate-NADPH. The fluorescence decay of the enzyme-bound NADPH is not monoexponential in any of these complexes. Moreover, it does not seem to be dependent on the coenzyme concentration. The experimental curves can be satisfactorily fitted with the sum of two exponentials, the relative amplitudes of which significantly depend on the complex studied. Thus, for dihydronicotinamide two possible environments might exist in the enzyme active sites. It is also shown that the fluorescence decay times of the enzyme are shortened by the bound NADPH.

Beef liver glutamate dehydrogenase, GDH[1] (EC 1.4.1.3), catalyzes reversibly the oxidative deamination of L-glutamate into 2-oxoglutarate. The GDH active unit consists of six polypeptide chains with the same primary sequence (Moon et al., 1972). GDH can utilize both NAD(H) and NADP(H) as coenzymes. However the behavior of the enzyme is less complex with NADPH (Frieden, 1963, 1971). It has been demonstrated that the reduced coenzymes have two binding sites on each protomer (Pantaloni and Dessen, 1969; Jallon and Iwatsubo, 1971). As far as NADPH is concerned, the affinity of the coenzyme for the active site is much higher than that for the regulatory site (Koberstein and Sund, 1973).

The catalytic and structural properties of GDH are modified by the binding of a number of ligands. For example, the allosteric inhibitor GTP induces depolymerization of the enzyme and stabilizes the coenzyme binding to the enzyme. The stability of this complex is also enhanced by the reduced substrate L-glutamate. Finally, it has been shown that a quaternary complex is formed with GTP and L-glutamate (Jallon and Iwatsubo, 1971, 1973).

NAD(P)H binding to GDH brings about marked changes in the absorption, circular dichroism, and fluorescence spectra of these coenzymes. Moreover, the fluorescence intensity of the reduced coenzyme is increased after binding. GTP and/or L-glutamate induce further changes of some of these properties (Winer and Schwert, 1958; Fisher and McGregor, 1960; Fisher and Cross, 1966; Di Franco and Iwatsubo, 1972; Jallon and Iwatsubo, 1971, 1973; Pantaloni and Lecuyer, 1973). Coenzymes also alter the circular dichroism of the enzyme aromatic residues (Jallon et al., 1973). The reduced coenzymes quench the enzyme intrinsic fluorescence. This phenomenon has been attributed to an energy transfer between tryptophan residues and dihydronicotinamides. In agreement with such an interpretation, Sommers and Yielding (1970) have found that the average decay time constant of NADH bound to GDH is longer when the exciting wavelength is situated in the enzyme absorption band than when it is in the dihydronicotinamide absorption band.

In the present paper, we report a pulse fluorimetry study of the ternary complexes GDH-NADPH-GTP and GDH-NADPH-L-glutamate and of the quaternary complex GDH-NADPH-GTP-L-glutamate. We used a single photon pulse fluorimeter which has a better sensitivity and better performances than those of the apparatus used by Sommers and Yielding (1970). Transient fluorescence obtained with this apparatus may be analyzed on the basis of decay curves which are exponential sums. Thus, a complex emission may be resolved into its components (Wahl and Auchet, 1972; Wahl and Brochon, 1973; Donzel et al., 1974; Wahl, 1975).

In this work, the proteinic complexes were excited within the NADPH absorption band at 336 nm. An analysis of the transient fluorescence obtained shows that the dihydronicotinamide ring environment is different in each of these complexes. Moreover, for each complex, this aromatic ring has at least two different environments.

We also report the fluorescence of the tryptophan, of the free enzyme, and of the complexes. The tryptophan fluorescence decays differ also from one complex to another; however, the fluorescence of all complexes is quenched relative to unliganded GDH. In the complexes bearing GTP, the fluorescence quenching cannot be due only to an excitation transfer.

Experimental Procedure

Beef liver GDH was prepared according to Kubo et al. (1958). The enzyme was stored at 4 °C as a crystal suspension in a 7.5% sodium sulfate solution. Before each series of experiments, the suspension was centrifuged, the pellet dissolved in the minimum buffer, and the mixture again centrifuged. NADPH (P-L Biochemicals) was purified by chromatography on DEAE-cellulose (Brochon, 1974). GTP (Sigma) and L-glutamate (Fluka A.G.) were used without further purification. The buffer was 0.1 M Tris-HCl-1 mM EDTA (pH 7.5) in twice distilled water. The following molar concentrations were determined spectrophotometrically: GDH protomer (mol wt 56 100) at 279 nm (ϵ = 0.97 cm^2 mg^{-1}); GTP at 252 nm (ϵ 13 600 M^{-1} cm^{-1}); NADPH at 340 nm (ϵ 6200 M^{-1} cm^{-1}). *p*-Terphenyl- and 1,1,4,4-tetraphenyl-1,3-butadiene have been obtained from Koch Light Laboratories.

Transient fluorescence curves have been measured with the photon counting method (Wahl, 1969; Wahl and Auchet,

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[1] Abbreviations used are: GDH, glutamate dehydrogenase; NADPH, reduced nicotinamide adenine dinucleotide phosphate; NADH, reduced nicotinamide adenine dinucleotide; GTP, guanosine triphosphate.

1972; Wahl, 1975). The photomultipliers used were the Radiotechnique 56 DUVP and RCA 8850, except for the measurement of the 1,1,4,4-tetraphenyl-1,3-butadiene time constant, for which the Radiotechnique PM 2 106 was used. The excitation light at 260, 295, and 375 nm was provided by a high-pressure H_2 flash lamp and a Bausch & Lomb monochromator (500 mm). The bandwidths were, respectively, 13, 6.5, and 10 nm. The excitation at 336 nm was obtained with a high-pressure N_2 flash lamp and an M.T.O. (M.T.O., Métallisation et Traitements Optiques, Massy, France) interference filter ($\Delta\lambda$ = 4 nm). For the emission at 464 nm, we used an interference filter M.T.O ($\Delta\lambda$ = 19 nm) associated with the following filters: Schott GG 395 and M.T.O J 385-a. The emissions at 350 and 425 nm were selected by a Bausch & Lomb monochromator high intensity. The bandwidths were, respectively, 13 and 10 nm. The flash frequency was around 10 kHz. Data collection was stopped when the total count in the fluorescence curve was about 1×10^6, which was reached in 2 h with the Radiotechnique PM's, where a counting rate of 1–2% of the flash frequency was maintained. The storage time could be reduced to 15 min with the PM 8 850 by using the amplitude selection of single photoelectron pulses (Miehe et al., 1970; Schuyler and Isenberg, 1971).

Transient Fluorescence Analysis. Experimental transient fluorescence curves correspond to convolution products such as:

$$i(t) = \int_0^t g(T)I(t - T)\mathrm{d}T \qquad (1)$$

where $g(t)$ is the apparatus response function; $I(t)$ is the fluorescence decay which would be the transient fluorescence obtained with an infinitely short excitation.

Determination of the Response Function. It has been shown that the response function $g(t)$ is dependent on the light wavelength impinging on the photomultiplier cathode (Wahl et al., 1974). According to the method of these authors, the true $g(t)$ was determined from the experimental transient fluorescence of a reference solution having a monoexponential decay. The excitation and emission spectra of the reference and studied solutions must overlap. The decay measurements must be performed under the same conditions for both solutions. In the present work the reference compound was *p*-terphenyl for emissions at 350 nm (decay time 0.96 ns; Wahl et al., 1974) and 1,1,4,4-tetraphenyl-1,3-butadiene in deaerated cyclohexane for emission at 464 nm. The decay time of 1,1,4,4-tetraphenyl-1,3-butadiene was determined with excitation and emission wavelengths set at 375 and 425 nm, respectively, and by using the fast photomultiplier PM 2 106. Under those conditions, the wavelength effect is small and $g(t)$ could be determined by using a scattering solution. Several determinations of the 1,1,4,4-tetraphenyl-2,3-butadiene decay time were made. The average value was 1.76 ± 0.02 ns at 20 °C and 1.78 ± 0.02 ns at 10 °C. These values are in perfect agreement with the values published by Berlman (1965) (1.76 ns). Details on such measurements are given in the Results section of this work.

Determination of the Decay Parameters. The fluorescence decay $I(t)$ (see eq 1) was assumed to be a sum of exponential functions which could be written:

$$I(t) = \sum_{i=1}^{p} A_i \exp(-t/\tau_i) \qquad (2)$$

where the τ_i were the decay times and the A_i the amplitudes of the exponential terms. In the following tables of results, we use the relative amplitude defined as follows:

$$C_i = A_i \Big/ \sum_{i=1}^{p} A_i$$

Contrary to the A_i's, the C_i's have an intrinsic meaning independent of the arbitrary number of counts used in the fluorescence measurements. For $p = 2$, one has evidently:

$$C_1 = 1 - C_2 \qquad (3)$$

p was successively taken equal to 1, 2, ... For each curve, one determined the parameters τ_i and A_i which gave the best fit to the transient fluorescence. A first estimation of these parameters was obtained by using a computer program based on the modulation function method (Valeur and Moirez, 1973). A set of time constant τ_i values was then tried in order to improve the mean weighted residue R defined by eq 4.

The amplitudes A_i were determined by the formula of cutoff moments (Valeur and Moirez, 1973). R is defined by the following expression:

$$R = \frac{1}{n} \sum_{i=1}^{n} \frac{(i_c{}^k - i_{ex}{}^k)^2}{i_{ex}{}^k} \qquad (4)$$

where $i_{ex}{}^k$ is the value of the count in the k^{th} channel of the transient fluorescence, $i_c{}^k$ is the value of the convolution product computed by formula 1, and n is the number of channels.

Another important way of appreciating the fit was to draw the deviation function (Grindwald and Steinberg, 1974). This function is defined by the following relation:

$$DV^k = \frac{i_c{}^k - i_{ex}{}^k}{(i_{ex}{}^k)^{1/2}} \qquad (5)$$

Photocounting obeys the Poisson statistics (Knight and Selinger, 1971). Then if we neglect the statistical error in the determination of $g(t)$, the optimum fit should be characterized by a value of R equal to one. In addition, the deviation function (eq 5) should fluctuate randomly around zero, with an average amplitude equal to one.

This ideal behavior has seldom been observed, which might be due to the following systematic errors: (1) reflections of electric fast impulses at cable junctures; (2) radiofrequency pick-up (Ware, 1971; Isenberg, 1975); (3) incomplete correction of the wavelength effect in the determination of $g(t)$, which could occur when the emission filter defined a wide wavelength band in which the spectrum shapes of the sample and the reference compound were sensibly different (P. Gauduchon and Ph. Wahl, unpublished results).

The statistical errors occurring in the determination of $g(t)$ might also increase the value of R and amplify the random oscillation of the deviation function. This effect could be shown to be important only when the time constants of the sample were smaller than the time constant of the reference compound (Ph. Wahl, manuscript in preparation).

Since it was not possible to eliminate completely all of these errors, one was led to define an optimum fit for given experimental conditions. This was obtained by measuring the transient fluorescence of a simple compound which was expected to be a single exponential. With such a compound, it was observed that a single exponential decay could be found with a value of R close to one. In addition, $DV(t)$ had a Poissonian behavior over a great portion of the exploration time range. Furthermore, R and $DV(t)$ were not greatly improved by using a sum of two exponential functions as decay function (see below and P. Gauduchon and Ph. Wahl, manuscript in preparation).

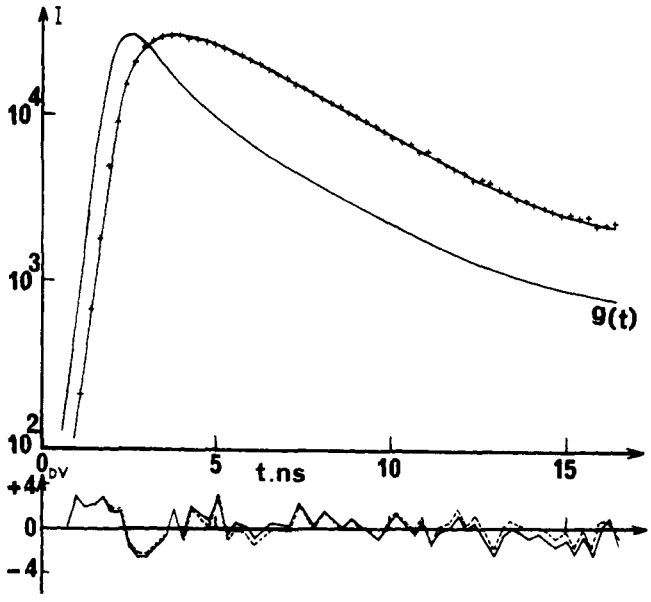

FIGURE 1: Transient fluorescence of 1,1,4,4-tetraphenyl-1,3-butadiene: (upper curves) fluorescence and $g(t)$; (lower curves) deviations relative (1) to a single exponential (—), $\tau = 1.79$ ns, $R_1 = 3.2$; (2) to a sum of two exponentials (-+-) $\tau_1 = 1.70$ ns, $\tau_2 = 2.8$ ns, $C_1 = 0.93$, $R_2 = 2.8$.

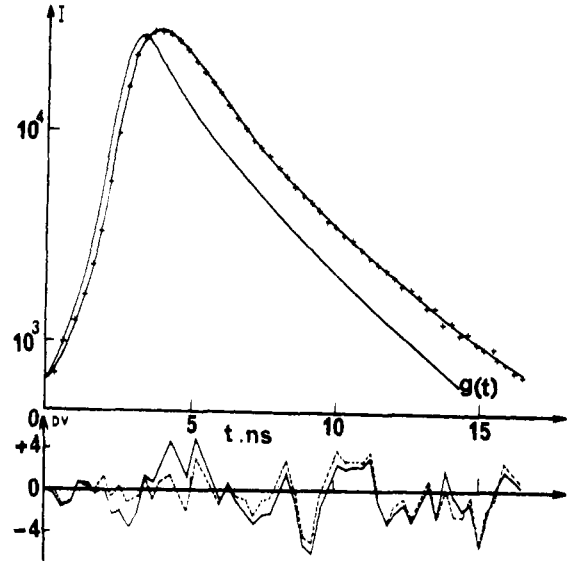

FIGURE 2: Transient fluorescence of free NADPH: (upper curves) fluorescence and $g(t)$; (lower curves) deviations relative (1) to a single exponential (—), $\tau = 0.62$ ns, $R_1 = 6.9$; (2) to a sum of two exponentials (-+-) $\tau_1 = 0.36$ ns, $\tau_2 = 0.83$ ns, $C_1 = 0.625$, $R_2 = 5.1$.

On the other hand, one could conclude that a fluorescence decay did not correspond to a single exponential whenever the best fit obtained with such a function was not as good as the optimum fit. In that case R was great and $DV(t)$ diverged systematically from zero. One tried then to approach the optimum fit with two exponential decays. In the following experiments, it was not found necessary to go beyond $p = 2$. Whenever a good fit could be obtained with a given value of p, it was generally possible to obtain also a good fit with $p = p + 1$. This means that p is the minimum number of time constants which were actually present in the decay.

For a set of chromophores each characterized by a decay time, τ_i, but having the same radiative rate constant, the quantum yield, Q, is related to the mean time constant of first order $\langle\tau\rangle$ by the equation:

$$Q = \langle\tau\rangle k_F \tag{6}$$

with

$$\langle\tau\rangle = \sum_i c_i\tau_i \tag{7}$$

where the c_i's are the exponential amplitudes.

Results

Transient Fluorescence of 1,1,4,4-Tetraphenyl-1,3-butadiene. The decay time was measured as described in the Experimental Procedure section. The residue values were around 2. In the case of the experiment shown in Figure 1, the value of R was 3.2. The minimum residue obtained with a biexponential decay was 2.8 with $\tau_1 = 1.70$ ns, $\tau_2 = 2.8$ ns, and $C_1 = 0.93$. The corresponding curves $DV(t)$ are also presented in Figure 1. These curves oscillate randomly about the time axis. It can be seen that the shapes of the deviation curves relative to one exponential and to two exponential decays are very similar. According to our preceding discussion, the decay of 1,1,4,4-tetraphenyl-1,3-butadiene can be considered as a single exponential decay.

Transient Fluorescence of Free NADPH. As another example of single exponential decay, we have studied the free NADPH dissolved in Tris buffer at 0 °C. The excitation wavelength was 260 nm. The coenzyme NADPH in aqueous solution presents two kinds of conformations (Jardetzky and Wade-Jardetzky, 1966) characterized by different decay times (Brochon, 1974). Under these conditions, the stacked form of the coenzyme only is excited (Weber, 1958). The apparatus response function was determined with 1,1,4,4-tetraphenyl-1,3-butadiene as a reference solution.

In the case of a single exponential decay, the best time constant was $\tau = 0.62$ ns with $R = 6.9$. In the case of a biexponential decay, we obtained a minimum residue of $R = 5.1$. The time constants were $\tau_1 = 0.36$ ns and $\tau_2 = 0.83$ ns, and the relative amplitude was 0.625. It can be seen in Figure 2 that the general shape of the deviation curves is similar for both decays. The small improvement of the residue value is associated with a small improvement of the deviation curve. In this case the amplitude of these deviations was relatively important. This was probably due to the amplification of the counting statistical errors which occurred when the sample had a decay time smaller than the reference solution (see the Experimental Procedure section). At 20 °C, the value of the decay time was 0.42 ns, close to the values measured by other authors (Scott et al., 1970; Schuyler et al., 1972).

Transient Fluorescence of GDH Complexes Excited at 336 nm. The measurements were performed for the ternary complexes GDH-NADPH-GTP and GDH-NADPH-L-glutamate and for the quaternary complex GDH-NADPH-GTP-L-glutamate at different temperatures (0-30 °C). While the concentration of enzyme protomers was 70 μM, that of the reduced coenzyme varied between 5 and 50 μM ([GTP] = 470 μM and [L-glutamate] = 50 mM).

For all the studied complexes, whatever the conditions, it was not possible to obtain a good fit of the transient fluorescence curves with a single exponential. But such a good fit was obtained with sums of two exponentials (See Table I). Examples of deviation curves are shown in Figures 3 and 4. The curves corresponding to the best single exponential diverge systematically from the time axis along the whole time range explored. On the contrary, for the two exponential decays, the deviation functions oscillate randomly. As shown in Figure 4,

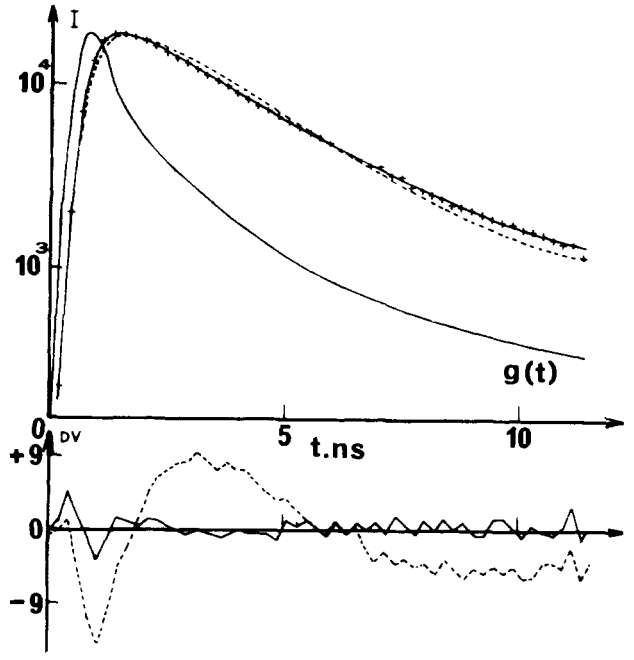

FIGURE 3: Transient fluorescence of NADPH bound to GDH-GTP complex. Reconstitution of the experimental curve (+) with convolution products assuming either a monoexponential decay (τ = 2.4 ns, R_1 = 29.8) or a biexponential decay (τ_1 = 1.44 ns, τ_2 = 4 ns, C_1 = 0.76, R_2 = 2.0.). The convolution and the deviation functions are represented with an interrupted line for the single-exponential decay and with an continuous line for the double-exponential decay. Excitation at 336 nm, emission at 464 nm; [GDH] = 70 μM; [NADPH] = 20 μM; [GTP] = 470 μM; t = 0 °C.

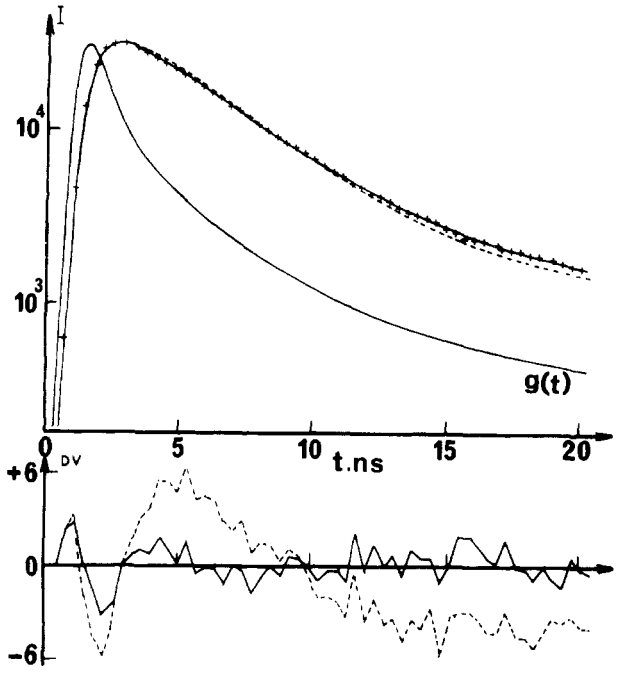

FIGURE 4: Transient fluorescence of NADPH bound in GDH-NADPH-GTP-L-glutamate complex. Reconstitution of the experimental curve (+) with convolution products, assuming either a monoexponential decay (τ = 2.18 ns, R = 14.0) or a biexponential decay (τ_1 = 1.85 ns, τ_2 = 4.0 ns, C_1 = 0.90, R = 2.4). The convolution and the deviation functions are represented with an interrupted line for the single-exponential decay and with an continuous line for the double-exponential decay. Excitation at 336 nm, emission at 464 nm; [GDH] = 70 μM; [NADPH] = 20 μM; [GTP] = 470 μM; [L-glutamate] = 50 mM; t = 0 °C.

TABLE I: Transient Fluorescence of Some GDH-NADPH Complexes; Compared Analysis as a Sum of Two Exponentials or a Single Exponential.[a]

GDH-NADPH +	Temp (°C)	[NADPH] (μM)	τ_1 (ns)	τ_2 (ns)	C_1	Residue
L-Glutamate	0	20	2.6		1	31
			1.67	3.4	0.60	3.8
	20	20	2.06		1	17.5
			1.20	2.6	0.52	2.3
GTP	0	20	2.40		1	29.8
			1.44	4.0	0.76	2.0
	20	20	2.03		1	24.5
			1.10	3.3	0.75	2.6
GTP + L-glutamate	0	20	2.18		1	14
			1.85	4.0	0.90	2.4
	20	20	1.78		1	18.3
			1.30	2.9	0.75	4.6

[a] The excitation and emission wavelengths are, respectively, 336 and 464 nm. Enzyme concentration: 70 μM; the GTP and L-glutamate concentrations were, respectively, 470 μM and 50 mM; 0.1 M Tris-HCl-1 mM EDTA (pH 7.5).

TABLE II: Fluorescence Decay Parameters of Ternary GDH-NADPH-GTP Complexes (Excitation at 336 nm, Emission at 464 nm).[a]

Temp (°C)	[NADPH] (μM)	$\langle\tau\rangle$ (ns)	τ_1 (ns)	τ_2 (ns)	C_1	Residue
0	10	2.01	1.44	4.3	0.80	3.8
	20	2.05	1.44	4.0	0.76	2.0
	30	2.05	1.44	3.8	0.74	6.3
10	5	1.82	1.25	6.0	0.88	2.9
	20	1.78	1.25	4.2	0.82	5.5
	30	1.74	1.20	3.9	0.80	4.7
20	10	1.69	1.1	3.6	0.764	3.7
	20	1.65	1.1	3.0	0.748	2.6
	30	1.63	1.0	3.0	0.683	5.8

[a] Other experimental conditions are as in Table I.

the long time constant of the quaternary complex is easily detected in spite of its small relative contribution which amounts to 10% only.

The whole set of data resulting from the decomposition into the sum of two exponentials is shown in Tables I-IV, together with the average time constants, calculated with formula 7. It is obvious that the decay parameters are different with each complex. For example, the relative amplitude C_1 is markedly higher in the complexes where GTP is present.

Generally speaking, no important systematic variations of the decays, with the coenzyme concentration, are observed.[2] The relative amplitude C_1 of the quaternary complex decreases with temperature. But, for both ternary complexes, C_1 is independent of the temperature. In agreement with formula 7, the average time constants of the various complexes are in the ratio of the published quantum yield values (Churchich, 1967; Di Franco and Iwatsubo, 1972; Jallon, 1974).

In particular the mean decay constant of the quaternary complex at 10 °C is significantly lower than that of the ternary complexes, which confirms the quantum yield measurements

[2] However, for the ternary complex with GTP, there is a small decrease of C_1, when the coenzyme concentration is increased.

TABLE III: Fluorescence Decay Parameters of Ternary GDH-NADPH-L-Glutamate Complexes (Excitation at 336 nm, Emission at 464 nm).[a]

Temp (°C)	[NADPH] (μM)	⟨τ⟩ (ns)	τ_1 (ns)	τ_2 (ns)	C_1	Residue
0	10	2.26	1.67	3.4	0.66	5.3
	20	2.36	1.67	3.4	0.60	3.8
	30	2.31	1.67	3.0	0.52	3.4
10	10	1.27	1.30	2.7	0.590	2.8
	20	1.92	1.4	2.7	0.596	3.0
	30	1.84	1.3	2.7	0.615	3.9
20	20	1.87	1.20	2.6	0.520	2.3
	30	1.95	1.35	2.6	0.517	3.2
30	10	1.99	1.4	2.6	0.51	6.7
	20	1.79	1.25	2.6	0.60	3.5
	30	1.77	1.30	2.6	0.64	2.7

[a] Other experimental conditions are as in Table I.

TABLE IV: Transient Fluorescence of Quaternary GDH-NADPH-GTP-L-Glutamate Complexes.[a]

Temp (°C)	[NADPH] (μM)	⟨τ⟩ (ns)	τ_1 (ns)	τ_2 (ns)	C_1	Residue
0	5	2.07	1.85	4.6	0.92	5.2
	20	2.10	1.85	4.0	0.90	2.4
	30	2.06	1.85	4.0	0.90	2.8
	40	2.0	1.85	3.8	0.92	9.8
10	5	1.64	1.20	3.6	0.82	1.6
	20	1.54	1.25	2.7	0.80	2.0
	30	1.47	1.25	3.0	0.83	2.6
	40	1.47	1.25	2.7	0.85	2.6
	50	1.48	1.25	2.7	0.84	2.8
20	20	1.70	1.30	2.9	0.75	4.6
	40	1.46	1.05	2.3	0.67	3.6
30	20	1.58	1.15	2.6	0.705	4.0
	40	1.53	1.15	2.3	0.673	5.7

[a] Other experimental conditions are as in Table I.

(Jallon, 1974). Finally, a discontinuity of the properties between 0 and 10 °C appears. This result recalls the abnormality in polymerization which has been recently pointed out (Thusius et al., 1975).

Transient Fluorescence Emission of the GDH Tryptophan Residues. Fluorescence decays of the enzyme without ligands and of the three complexes studied were measured with an excitation wavelength of 295 nm and an emission wavelength of 350 nm. Under these conditions, the contribution of tyrosines to emission can be neglected. We verified that the stray light was negligible, using a ludox solution which had the same scattering intensity at 330 nm as the GDH solution. The NADPH concentration was chosen such that all the enzyme active sites were occupied. According to various authors, the dissociation constant of the enzyme-NADPH complex lies between 0.6 and 3 μM for the ternary complex with GTP, between 0.5 and 2 μM for the ternary complex with L-glutamate, and is close to 0.01 μM for the quaternary complex (Di Franco and Iwatsubo, 1972; Koberstein and Sund, 1973). It should be noted that under these conditions, dissociation of the polyhexamer is complete in the complexes with GTP (Frieden, 1971; Dessen and Pantaloni, 1973).

Experimental decay curves are shown in Figure 5. We obtained a good simulation of the experimental curves with a sum

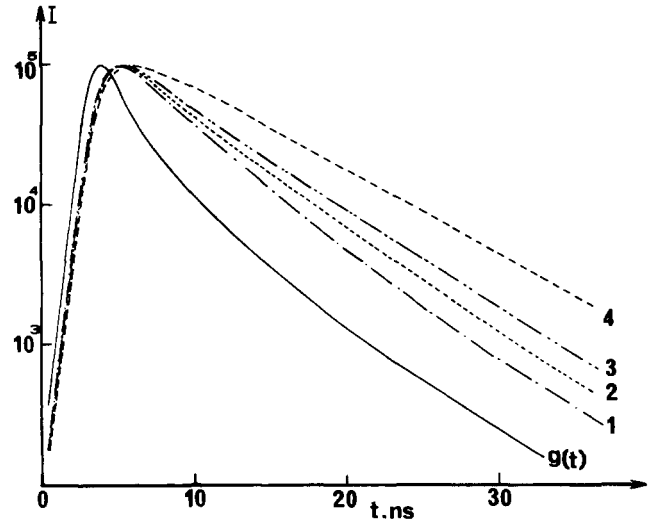

FIGURE 5: Transient fluorescence of GDH tryptophans in various complexes: (1) GDH-NADPH-GTP; (2) GDH-NADPH-GTP-L-glutamate; (3) GDH-NADPH-L-glutamate; (4) GDH without any ligand. Excitation and emission wavelengths are, respectively, 295 and 350 nm. Experimental conditions are as described in Table V.

of two exponentials determined by the modulating function method. The data are gathered in Table V. We notice that the NADPH binding considerably decreases the two decay times τ_1 and τ_2 and their average ⟨τ⟩. This phenomenon is more marked when GTP is present. Moreover, the amplitude of τ_1 increases. The value of ⟨τ⟩ (4.53 ns) for the fluorescence decay of the unliganded enzyme is comparable to the value obtained by Sommers and Yielding (1970) with the same excitation and emission wavelengths.

Discussion

The study of the binding equilibria of NADH and NADPH to the two complexes, enzyme-GTP and enzyme-L-glutamate, shows the existence of two types of coenzyme binding sites per protomer: the active sites and the regulatory sites (Frieden, 1963; Jallon and Iwatsubo, 1971; Koberstein and Sund, 1973). As far as NADPH is concerned, the dissociation constants for these two sites are about 2 and 600 μM at 20 °C (Koberstein and Sund, 1973) in the absence of any other ligand. The published data show that the affinity for the regulatory site is neither sensitive to the presence of GTP nor of L-glutamate (Koberstein and Sund, 1973; Jallon, 1974).

In our transient fluorescence measurements with excitation at 336 nm, the coenzyme molar concentration was not higher than 70% of the enzyme protomer concentration. Under these conditions, coenzyme binding can occur only at the active site. Moreover, the polarization degree of the fluorescence of the bound coenzyme is high, indicating that the nicotinamide ring is rigidly bound to its site. This implies that its excitation does not affect its position.

However, the fluorescence decay is not exponential as it should be if all the dihydronicotinamide rings had the same environment. Thus, one has to postulate the existence of several possible environments for the nicotinamide ring. Whichever the complex, we could not detect any significant dependence of the decay parameters with the coenzyme concentration. This suggests that the association equilibria of the coenzyme with these complexes are not directly related to the nicotinamide interaction with its proteinic environment. The important interactions would then involve the other parts of NADPH, that

TABLE V: Transient Fluorescence of GDH Tryptophanyl Residues in Various Complexes.[a]

GDH	[NADPH] (μM)	$\langle\tau\rangle$ (ns)	τ_1 (ns)	C_1	τ_2	C_2	Residue
		4.53	2.07	0.412	6.25	0.59	1.7
+ NADPH + GTP	76	1.80	1.25	0.771	3.65	0.229	1.6
+ NADPH + L-glutamate	47	2.54	1.50	0.720	5.20	0.280	2.1
+ NADPH + GTP + L-glutamate	47	2.13	1.36	0.763	4.60	0.237	1.5

[a] Excitation at 295 nm, emission at 350 nm; [GDH] = 32 μM; [GTP] = 470 μM; [L-glutamate] = 48 mM; t = 10 °C.

is to say adenine, riboses, and phosphates. This recalls the crystallographic data obtained with the lactate dehydrogenase (Adams et al., 1973) and the nuclear magnetic resonance (NMR) study of the GDH-NADPH interaction (Roux and Jallon, 1974). The existence of several environments around the nicotinamide could proceed from different coenzyme conformations or from different configurations of its proteinic subsites. Our experiments do not allow a choice between these two interpretations. The decomposition into a sum of two exponentials gives satisfactorily results, which suggests that there are only two distinct environments for NADPH in each complex.

One has to mention that the main difference between the three complexes is the relative number of molecules in each environment (determined by C_1). If one assumes that the two states of the bound coenzyme are in equilibrium and have the same absorbance at the excitation wavelength, the equilibrium constant is equal to $(1 - C_1)/C_1$. This constant does not vary markedly with temperature except for the quaternary complex. It depends on the nature of the complex; it reaches its maximum value in the absence of GTP. For example at 10 °C, it is 0.67 for enzyme-NADPH-L-glutamate and 0.2 for enzyme-NADPH-GTP and the quaternary complex. The proportion of these two coenzyme states might be important to control the enzyme efficiency and thus regulate it.

Finally, we found that the fluorescence decay times of the enzyme-NADPH complexes are shorter than the decay times of the enzyme without NADPH. These results agree with the intensity measurements under steady excitation light, which show that the intrinsic fluorescence of the enzyme is quenched by NADPH binding (Di Franco and Iwatsubo, 1972).

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Effect of Polylysine on the Activation of Prothrombin. Polylysine Substitutes for Calcium Ions and Factor V in the Factor Xa Catalyzed Activation of Prothrombin†

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ABSTRACT: Polylysine has been demonstrated to dramatically accelerate the rate of the factor Xa catalyzed activation of both prothrombin and prethrombin 1. Under the present experimental conditions (pH 8.0, 23 °C), no detectable activation of prothrombin or prethrombin 1 occurs with either factor Xa or polylysine alone. The activation of prethrombin 2, the direct precursor of α-thrombin, by factor Xa is not stimulated by polylysine. The activation of either prothrombin or prethrombin 1 by factor Xa in the presence of polylysine is partially inhibited by the presence of 5 mM $CaCl_2$. Electrophoretic analysis in sodium dodecyl sulfate showed that the products that were formed in the above activation system comigrated with the reaction products derived from prothrombin activated by factor Xa in the presence of calcium ions and phospholipid. It is suggested that polylysine stimulates the factor Xa-catalyzed activation of prothrombin by replacing the combination of calcium ions and factor V.

The activation of partially purified equine prothrombin by polylysine was first described by Miller (1960). Subsequent work from that laboratory showed that a variety of polymeric amines could also activate these prothrombin preparations (Miller et al., 1961). Subsequently Aronson and Menáché (1968) showed that the activation of more highly purified prothrombin preparations by polylysine was dependent on the addition of factor Xa.[1]

The results from a number of laboratories have shown that the activation of prothrombin is a complex process involving one or two intermediate steps (Mann et al., 1971; Stenn and Blout, 1972; Heldebrant and Mann, 1973; Engel and Alexander, 1973; Owen et al., 1974; Kisiel and Hanahan, 1974; Silverberg and Nemerson, 1975). In view of these recent observations, the effect of polylysine on the factor Xa catalyzed activation of prothrombin and prothrombin activation intermediates was subjected to further investigation.

The present results suggest that polylysine can substitute for calcium ions and factor V in the factor Xa catalyzed activation of prothrombin.

Materials and Methods

Poly(L-lysine) hydrobromide samples of various molecular weight ranges were obtained from Miles-Yeda Laboratories, Inc. The molecular weights given in the text for these samples were taken from the manufacturer's data sheet as obtained by ultracentrifugal analysis. As these materials are quite hydroscopic, stock solutions of 10 mg/ml in distilled water were prepared utilizing the entire contents of a vial and stored at −20 °C until use. Bovine fibrinogen was purchased from Sigma Chemical Corp., while *p*-tosyl-L-arginine methyl ester (TosArgOMe)[2] was a product of Schwarz-Mann. Crude Russell's viper venom (Lot No. 04158-2) was obtained from Pierce Chemical Co. and used without further purification. Sodium [^{3}H]borohydride (6.4 Ci/mmol) was purchased from Amersham Searle. All other chemicals were of reagent grade and used without further purification.

Bovine prothrombin was prepared as described by Bajaj and Mann (1973). Prethrombin 1[3] and prethrombin 2 were prepared as previously described (Heldebrant et al., 1973). Prothrombin and the intermediates of prothrombin activation was dialyzed against 0.01 M NH_4HCO_3 and lyophilized prior to use. Bovine factor X_1 was purified as described by Fujikawa and co-workers (Fujikawa et al., 1972a) and was a generous gift of Drs. K. Fujikawa and E. W. Davie. Factor Xa was obtained by the activation of purified bovine factor X_1 by Russell's viper venom (Fujikawa et al., 1972b).

Fibrinogen-clotting activity and esterase activity were measured as previously described (Lundblad, 1971). Fibrinogen-clotting activity is expressed in terms of NIH units using

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[1] The nomenclature used for the various coagulation factors is that adopted by the International Committee on Thrombosis and Hemostasis.

[2] Abbreviations used are: TosArgOMe, α-*N*-*p*-toluenesulfonyl-L-arginine methyl ester; Tris, 2-amino-2-hydroxymethyl-1,3-propanediol.

[3] The nomenclature used for the polypeptide fragments derived from prothrombin activation is that recently developed by the International Committee on Thrombosis and Hemostasis (Paris, July, 1975). The reader is referred to a recent review (Mann, 1976) for a discussion of the nomenclature.