THE INVESTIGATION OF INTERACTIONS IN THE EXCITED STATE OF FLAVINS USING TIME-RESOLVED SPECTROSCOPY

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Het werk, dat in dit proefschrift is beschreven, is uitgevoerd onder leiding van de promotor en stond gedeeltelijk onder de supervisie van Prof.dr. J.D.W. van Voorst en van Dr.F. Müller.

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A.J.W.G. Visser

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ter verkrijging van de graad van
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hoogleraar in de virologie,
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Dit proefschrift met stellingen van Antonie Jan Willem Gerrit Visser, doctorandus in de Chemie, geboren te Voorburg op 29 mei 1943, is goedgekeurd door de promotor, dr. C. Veeger, hoogleraar in de biochemie.

De Rector Magnificus van de Landbouwhogeschool
J.P.H. van der Want

Wageningen, 29 januari 1975

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STELLINGEN

1

De suggestie van Penzer *et al.* dat de pH-afhankelijkheid van de fotoreductiesnelheid van flavines kan worden toegeschreven aan een flavin-triplet met een pK_a-waarde van 5.7, moet van de hand worden gewezen.

G.R. Penzer, G.K. Radda, J.A. Taylor, M.B. Taylor, "Vitamins and Hormones" Vol. 28, p. 441 (R.S. Harris, P.L. Munson and E. Diczifalusy, eds), Academic Press, New York, 1970.
Dit Proefschrift.

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Het mechanisme, dat Kotanigawa en Simokawa voorstellen voor de specifieke ortho-alkylering van fenol is onwaarschijnlijk.

T. Kotanigawa en K. Shimokawa, Bull. Chem.Soc. of Japan 47(4) (1974) 950 en 47(6) (1974) 1535.

3

Bij de toepassing van fluorescente "probes" in biologische systemen verdient het aanbeveling zich vooraf op de hoogte te stellen van de intrinsieke moleculair-fysische eigenschappen van de "probe". Daarmee kunnen verkeerde interpretaties, zoals voorkomende in het werk van Weltman *et al.*, worden voorkomen.

J.K. Weltman, R.P. Scaro, A.R. Frackelton, R.M. Dowben, J.R. Bunting en R.E. Cathou, J.Biol.Chem. 248 (1973) 3173.

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De waargenomen snelheidsconstanten voor associatie van bepaalde repressor-operator systemen zijn onwaarschijnlijk hoog.

A.R. Riggs, R.F. Newby en S. Bourgeois, J.Mol.Biol. 51 (1970) 303.

5

Het door Dubourdieu *et al.* voorgestelde schema voor de endotherme bindingsreactie van FMN aan het *Desulfovibrio vulgaris* apoflavodoxine is niet in overeenstemming met de door hen gepresenteerde experimentele gegevens.

M. Dubourdieu, M.L. MacKnight en G. Tollin, Biochem.Biophys.Res.Commun. 60 (1974) 649.

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Inoue *et al.* dienen onderscheid te maken tussen het effect van de oligomeergrootte van het manteleiwit en van de tyrosine-modificatie op de initiatie- en de elongatiereacties van Tabaksmozaiek virus.

H. Inoue, K. Kuriyama, T. Ohno en Y. Okada, Arch.Biochem.Biophys. 165 (1974) 34.

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Birks en King stellen de vormingssnelheid van het 1,6-dimethylnaftaleen excimeer ten onrechte gelijk aan de klassieke botsingssnelheid, waarbij elke botsing tot reactie leidt.

J.B. Birks en T.A. King, proc.Roy.Soc. A 291 (1966) 244.

8

De veronderstelling dat de omzetting van 3-morfolino,5-oxo,6-fenyl,4,5-dihydro,1,2,4-triazine met fenylmagnesiumbromide tot 3-morfolino, 5,6-difenyl,1,2,4-triazine via een niet-cyclisch intermediair verloopt, is aan bedenkingen onderhevig.

A.K. Mansour en J.A. Ibrahim, J.Prakt.Chem. 315 (1973) 221

9

Song's bewering, dat de zwakke fosforescentie van flavines kan worden verklaard door een energiebarrière voor de intersystem-crossing aan te nemen, is in tegenspraak met de door hem uitgerekende grote spin-baan koppelingstermen.

P.S. Song, "Flavins and Flavoproteins" , p. 37 (H. Kamin, ed.), University Park Press, Baltimore, 1971.

10

Een centrale, goed geoutilleerde electronische en fijnmechanische werkplaats op de Dreyencomplex is uit practisch en economisch oogpunt te prefereren boven de huidige verspreiding van deze afdelingen over een aantal vakgroepen.

Proefschrift van A.J.W.G. Visser.

Aan mijn grootmoeder,
mijn ouders,
mijn vrouw en mijn kinderen

VOORWOORD

Vele mensen zijn op enigerlei wijze bij het tot stand komen van dit proefschrift betrokken geweest. Het betreft vele namen, niet alleen van vakgenoten, technisch en administratief personeel verbonden aan de drie laboratoria waar ik heb gewerkt, maar ook van mensen buiten het vakgebied, die mij tot grote steun zijn geweest. Zij allen hebben een belangrijke bijdrage geleverd tot mijn wetenschappelijke vorming en zij hebben de voltooiing van dit proefschrift mede mogelijk gemaakt. Zonder deze lange reeks namen op te noemen wil ik volstaan met het bedanken van alle betrokkenen voor hun onschatbare hulp.

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INTRODUCTION AND SUMMARY

Flavins are widespread in nature. As well known they are involved in metabolic oxidation-reduction and in biological electron transport processes |1, 2|. Flavins in the excited state play an important part in photobiology (phototropism and photoreception) as well as in many "in vitro" photoreactions. Several review articles dealing with this subject appeared in the literature |3, 4|. An attractive working hypothesis of these photoreactions is that they might mimic the enzymatic reactions catalyzed by flavoproteins. The flavin bound to the enzyme can be activated to react with substrates by conformational transitions induced in the protein after the binding of the substrate. In photoreactions the flavin can overcome the energy barrier by absorption of light, so that the flavin might reach an energetic higher metastable state from which reactions might occur. The anaerobic photoreactions can be divided into photoreductions, in which the substrate is oxidized and the flavin reduced, and in photoadditions where groups are substituted into a light excited flavin nucleus. These reactions are classified in the literature |4, 5|. In many cases the reduced flavin can be reoxidized by admitting air or oxygen to the solution. The properties of these photoreactions are specifically determined by the primary photochemical steps, i.e. the sequence of events occurring after excitation with an imaginary infinitely-small light pulse (δ-pulse).
The purpose of this thesis was to obtain informations concerning the fate of light excited flavin molecules. Most of the experimental and theoretical methods, results and discussions are already published in four articles, which form the basis of this thesis. This introduction is ment to give a short and general outline of the most significant results described in these publications. Two appendices containing some theoretical background are added. At the end of this thesis a short discussion is incorporated.

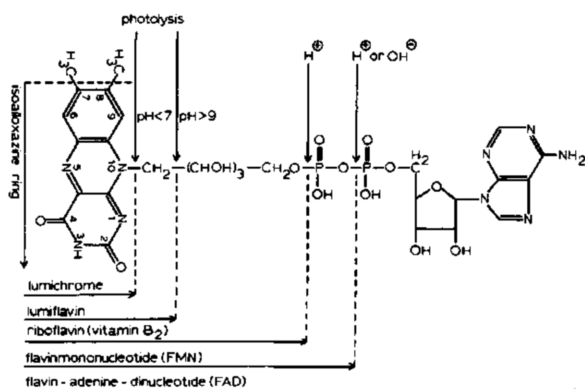

fig. 1
Structure and products of hydrolysis and photolysis of flavin adenine dinucleotide.

In order to study these photochemical and photophysical processes some advanced electronical and optical techniques were used, which have the advantage of an improved resolution in time, making measurements in the (sub)nanosecond time range possible. Some excellent papers describing these techniques appeared in literature. Therefore it is not necessary to mention experimental details, but the relevant literature is quoted |6-9|.
The flavins used in this work are represented schematically in figure 1. As can be seen from the figure the main part of the molecule, in which all the electronic properties are embodied, is the isoalloxazine ring. This aromatic moiety is in no way different from other aza-aromatic molecules.
The position of the electronic energy levels can be schematically represented in a Jablonski diagram (figure 2).

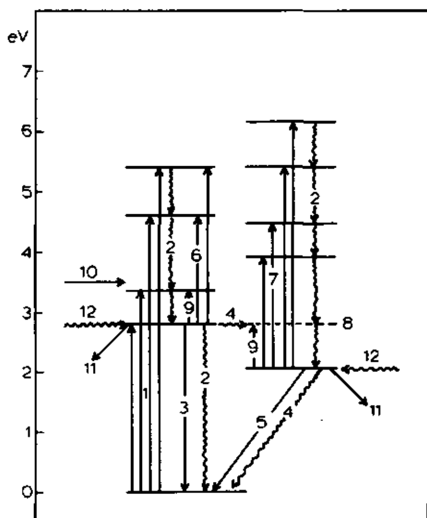

fig. 2
Jablonski-diagram describing the energy levels of singlet and triplet manifold of 3-methyllumiflavin; radiative and radiationless transitions are indicated; vibrational sublevels are not drawn; 1 eV = 8066.0 cm^{-1}; 1) absorption of light: S_0-S_n, n ≥ 1; 2) internal conversion; 3) fluorescence: S_1-S_0; 4) intersystem crossing; 5) phosphorescence; 6) laser photolysis: S_1-S_n absorption; 7) laser photolysis: T_0-T_n absorption; 8) virtual energy level calculated by Song |10|; 9) not observed transitions; 10) laser wavenumber; 11) photochemical reactions; 12) population of excited states via radiationless energy transfer.

There is much debate about the nature (n, π^* or π, π^*) of the excited singlet or triplet energy levels. A more complete discussion regarding this can be found in the literature |10, 11|. Also useful information concerning the direction of transition moments, oscillator strengths, charge density in the electronic states and spin density in the triplet state can be found in these references.
The possible pathways of excitation and de-excitation are also indicated in figure 2. The distribution of excitation energy over the molecular energy levels can be described by a multistep process |12|. The main steps are summarized below:

1. A flavin molecule in the ground state S_0 can be excited very rapidly (< 10^{-15} sec) by absorption of a quantum light to a higher vibronic singlet $S_{i,v}$ (i and v can be denoted as the quantum numbers for the electronic and vibrational state of the excited molecule).
2. From $S_{i,v}$ the molecule relaxes in a very short time (< 10 ps) to $S_{1,0}$ (internal conversion).
3. Radiationless deactivation (internal conversion) to the ground state $S_{0,0}$ is possible, but not very efficient because of the large energy gap between S_1 and S_0.
4. A quantum of light can be emitted (fluorescence), in which the emission lifetime is reflected by the lifetime of the excited state $S_{1,0}$ (in the order of 10 ns).
5. Intersystem crossing can occur from $S_{1,0}$ (or from a higher vibronic singlet) to an isoenergetic vibronic triplet state $T_{i,v}$ (in the order of 1 ns).
6. Internal conversion from $T_{i,v}$ to $T_{0,0}$ occurs in a very short time (< 10 ps).
7. From the metastable state $T_{0,0}$ the energy can be dissipated by light emission (phosphorescence) or radiationless to the ground state S_0 (1 μs - 1 s).

Apart from these intramolecular de-excitation pathways (2-7) several other steps originating from intermolecular interaction can be incorporated.

8. Because of its long lifetime and more favourable electronic distribution the triplet energy of the flavin is used as the driving force for chemical reactions, in which the flavin itself becomes reduced and the substrates become oxidized.
9. The flavin can accept energy from a higher excited energy level of another molecule (donor). This radiationless energy transfer might take place from donor singlet to acceptor singlet, from donor triplet to acceptor singlet or from donor triplet to acceptor triplet. In some cases the donor - acceptor interaction can be so strong (strong coupling) that the transfer rate is in the same order as or larger than the internal conversion or intersystem crossing (steps 2 and 5) |13, 14|. This so called before relaxation transfer, often leading to exciton states, has been ignored in the present work.
10. Short distance interactions with other molecules increase the probability of radiationless deactivation, which can be quantitatively measured via quenching of the fluorescence, phosphorescence or triplet-triplet absorption. As a consequence photoreactions mediated by the triplet state proceed at a lower rate. On the other hand the flavin forms weak complexes via Van der Waals-interaction with compounds like other flavins or aromatic electron donating molecules (e.g. phenols) |15-17|. If these "complexes" are present reactions might occur via the much shorter living excited singlet state.

For a better knowledge of the role of flavins in the excited state the scheme proposed in figure 2 is very convenient and in all four papers several examples are given which emphasize the usefulness of the scheme. The processes which were studied have also been depicted in figure 2. The papers are numbered as follows:

I Laser photolysis of 3-methyllumiflavin.

II Time-resolved fluorescence of FAD.

III Intrinsic luminescence studies on the apoenzyme and holoenzyme of lipoamide dehydrogenase.

IV A pulse fluorometry study of lipoamide dehydrogenase.

In paper I the results obtained with a very short (3 nsec) intense laser pulse as excitation source are described. This pulse excites such a large amount of flavin molecules into higher excited singlet and triplet states that changes in absorption of these higher excited states can be analyzed with a second less intense light flash of longer duration (5 μsec) fired synchronously with the laser pulse. The time-resolved absorption spectra obtained in this way were analyzed very carefully. When the pH was varied it was concluded that at 50 nsec after the laser pulse at least two species were present. One species could be identified as a flavin triplet. From photochemical and other data a structure for the second species was proposed, assuming the presence of flavin dimers. In such a flavin complex a very fast photoreaction occurs resulting in a photodimer, which is stable only in the excited state.
In paper II another pulse technique (single photon counting) was applied to the free nucleotides FMN and FAD. These flavins were excited with a repetitive cycle of short (2 nsec) weak light flashes and the fluorescence response was measured as a function of both wavelength and time. From these time-correlated fluorescence spectra several conclusions regarding the interaction of adenine and flavin moieties (in FAD) were drawn. The most important outcome was that only the open conformation of FAD is fluorescent. An appendix (1) is added, which treats the theory and method of the time-resolved fluorescence relevant to the FAD-system.
Paper III describes the results obtained with a flavoprotein (lipoamide dehydrogenase). Especially energy transfer experiments from tryptophan residues to the flavin indicate a crude distance and relative orientation between the 2 chromophores. In this study the time dependence of the tryptophan (donor) fluorescence was important. This was obtained from the phase delay of the fluorescence response on sinusoidally modulated exciting light.
Paper IV gives independent evidence from the single photon counting method that the distance between a tryptophan-flavin couple is in the same order of magnitude as calculated from static analysis (paper III). Furthermore the experiments indicate that not all the tryptophans are involved in energy transfer, a result also found in paper III. The formulas describing the fluorescence decays of donor and acceptor molecules are summarized in appendix 2. A close examination of the flavin fluorescence decay demonstrates that the two FAD's occupy different sites in the dimeric protein. Several experimental tests for establishing this inequality are treated in appendix 1. The most interesting observations and significant results are discussed at the end of this thesis.

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LASER PHOTOLYSIS OF 3-METHYLLUMIFLAVIN

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Abstract—The pH dependence of the transient absorptions of 3-methyllumiflavin has been investigated with laser photolysis in the nanosecond time range. Three different transient species (I, II and III) can be observed. The analysis of the decay curves shows that I and II can be ascribed to the optical absorption spectra of the lowest excited singlet and triplet states, respectively. Their appearance is not affected by a change of pH of the solution. The transient spectra due to species III have a decay which is dependent on pH. By comparison of these results with spectral data obtained with flavin dissolved in polymethylmethacrylate and with photochemical data reported in the literature, a structure for III is proposed which might explain the observed pH dependence.

INTRODUCTION

Flavins in the excited state have been shown to sensitize reactions involving a large number of biologically important compounds. A considerable number of these *in vitro* photoreactions has been published. A survey of the photochemical and photophysical properties of flavins is presented in several review articles (Penzer *et al.*, 1970; Song, 1971).

One aspect of these photoreactions is the influence of pH on the reaction rate (Penzer *et al.*, 1970; Song, 1971). In an attempt to clarify this point, a flash photolysis study was performed concerning the influence of pH upon the appearance of transient absorptions after flashing 3-methyllumiflavin in aqueous solution (Katan *et al.*, 1971). It was concluded that a 6 μs photoflash creates at least two different transient absorbing species. The absorption of one of these is affected by a change in pH.

This paper is a continuation of the previously reported flash-photolysis study. The extension reported here is essentially a better time resolution obtained by applying short laser pulses for the excitation of the flavin, which brings the time scale of the experiments into the nanosecond region.

MATERIALS AND METHODS

3-Methyllumiflavin was a gift from Professor P. Hemmerich, Konstanz. Other flavins used in this work were synthesized according to the literature (Hemmerich *et al.*, 1956; Ehrenberg *et al.*, 1967; Müller, 1964). Flavin mononucleotide (FMN) was obtained from Sigma Chemical Co.

The buffers were prepared as described previously (Katan *et al.*, 1971). A 1 cm^2 quartz cuvette attached to a glass bulb by means of a side arm, which can be connected to a vacuum line was used. The incorporation of 3-methyllumiflavin in polymethylmethacrylate plastic was performed as described elsewhere (Sun *et al.*, 1972). For flash photolysis experiments, polished polymethylmethacrylate rods (length 7 cm, dia 1·5 cm) were prepared. Polished blocks (13 × 13 × 10 mm) of the same material were used for phosphorescence or laser experiments. The flash apparatus has been described previously (Katan *et al.*, 1971).

Phosphorescence measurements were performed with a very sensitive phosphorimeter described earlier (Langelaar *et al.*, 1969). A CAT (Varian C-1024) was used to improve the signal-to-noise ratio in the phosphorescence lifetime measurements. A complete description of the instrumental operations for the laser photolysis experiments has been given elsewhere (Lavalette *et al.*, 1971; Bebelaar, 1974). Either frequency-doubled ruby (28,800 cm^{-1}) or frequency-tripled Nd^{3+}/glass (28,300 cm^{-1}) laser pulses of 3 ns duration were used for excitation of the flavin. The analyzing source was a doubly pulsed xenon discharge lamp giving light with a constant intensity during the first 5 μs after the laser pulse.

The time-resolved spectra were obtained from the oscillograms taken at wavenumber intervals of 500 cm^{-1}. For measurements at wavenumbers lower than 16,000 cm^{-1} a red sensitive photomultiplier (RCA C31025C) was used in the detection system. At each shot the integrated laser output was measured and the spectra were corrected for fluctuations in laser energy. Normally for each run a laser energy of about 5 mJ was employed. Photodestruction of the flavin was found to be insignificant as judged from the stationary absorption spectra recorded on a Cary 14 spectrophotometer taken before and after about 50 laser pulses.

All measurements were conducted at room temperature.

RESULTS

Laser experiments with aqueous solutions of 3-methyllumiflavin

Figure 1a shows the change in the absorption spectrum of buffered solutions of 3-methyllumiflavin at pH 5·6 and higher pH values at different times after excitation by the laser pulse (half-width 3 ns). The permanent absorption at 22,400 cm^{-1} (446 nm) is bleached, which results in a negative change in the absorption. New absorption maxima are observed in the region from 12,000 to 20,000 cm^{-1} and above 25,000 cm^{-1}. On a short time scale there is a small but rapid change in the transient absorption in the region from 27,000 to 30,000 cm^{-1} and below 14,000 cm^{-1} (transient I). Its decay time is almost equal to that of the fluorescence decay (about 5 ns). The remaining part of the transient absorption is characterized by a relatively slow decay in the μs time region (transient II).

In buffered aqueous solutions of 3-methyllumiflavin at pH values of 4·4, 3·0 and 1·8 the time development of the transient spectra observed after laser excitation differs from those obtained at pH values of 5·6 and higher by an additional rapid decay (transient III) in the region from 25,000 to 29,000 cm^{-1}, which is superimposed on that of transient I (27,000–30,000 cm^{-1}) (Fig. 1b).

Figure 2a gives a more detailed picture of the decay of the transient absorption at 25,000 cm^{-1} at pH 2·9. The decay is exponential (Fig. 3) and has a characteristic time (τ) of 52 ns at pH = 4·4, 40 ns at pH = 3·0, and less than 3 ns at pH = 1·8. The time-resolved absorption at 28,000 cm^{-1} shows (Fig. 2b) that the more rapidly decaying transient I is superimposed on transient III decaying with τ = 40 ns at pH = 3·0. For comparison the corresponding transient absorptions at pH 6·6 are given in Fig. 2c and d, respectively.

Figure 3 gives the decay of the transient absorption at 25,600 cm^{-1} as a function of pH. It is seen that at pH values < 5 the amount of transient III decreases with decreasing pH. The remaining part (superimposed on transient II and the rest of III) is representative for the change in intensity of the absorption at 25,600 cm^{-1} (390 nm) with pH as obtained in the photoflash experiments at 6 μs after the flash as described previously (Katan *et al.*, 1971).

From knowledge of the extinction coefficient of the transient absorption at a particular

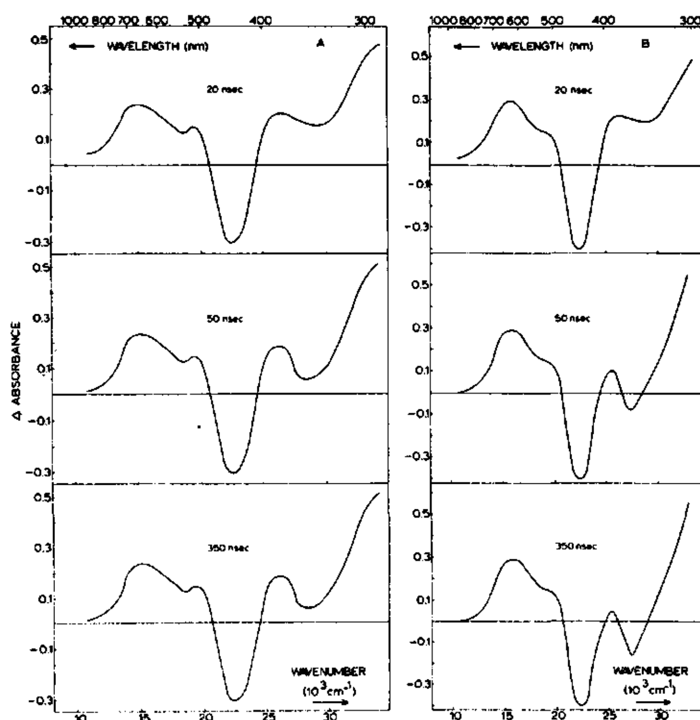

Figure 1. Transient absorption difference spectra at different times after the laser pulse. a: 105 μM 3-methyllumiflavin in 0·1 M phosphate buffer pH 7·0. b: 127 μM 3-methyllumiflavin in 0·1 M glycine–HCl buffer pH 2·9.

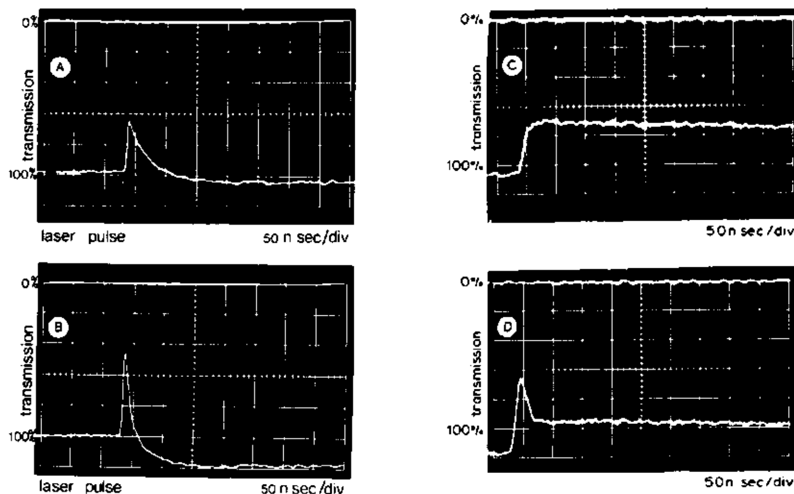

Figure 2. Oscilloscope traces of the decay of the transient absorption at different wavenumbers and at different pH values, flavin concentrations about 100μM. a: 25,600 cm^{-1}, pH 2·9; b: 28·000 cm^{-1}, pH 2·9; c: 25,600 cm^{-1}, pH 6·6; d: 28,000 cm^{-1}, pH 6·6.

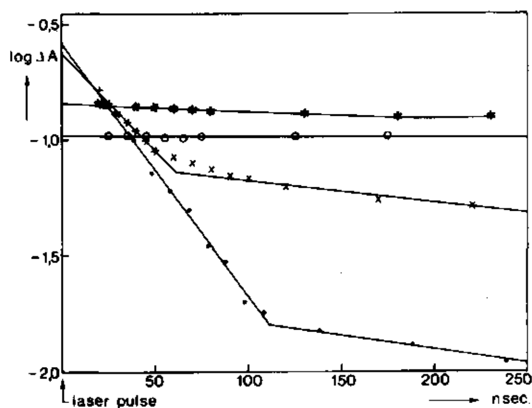

Figure 3. Semilogarithmic plot of the initial decay of the transient absorption at 25,600 cm^{-1} at ..... pH 3·0; × × × × × pH 4·4; ***** pH 5·6; OOOOO pH 7·9.

wavenumber, and from the ground-state absorption spectrum, the absolute transient absorption spectrum can be calculated. From a previous study the extinction coefficient (ϵ) at 15,000 cm^{-1} was found to be 4,700 l mol^{-1} cm^{-1}, which is of the same order of magnitude as found by Knowles and Roe for lumiflavin (ϵ_{680nm} = 4,600) (Katan *et al.*, 1971; Knowles and Roe, 1968; Vaish and Tollin, 1970).

Taking this into account it can be concluded that under certain conditions (high laser energy and perfect optical alignment) each shot converted about 60 per cent of the 3-methyllumiflavin present into transients II and III. On the other hand, after about 100 shots each with an average energy of about 5 mJ, only 1 per cent of the flavin was decomposed. From this it can be concluded that the process is almost quantitatively reversible.

It should be noted that all three transients are present directly after the laser pulse and that only a decay in transient absorption intensity has been observed.

Effect of oxygen

It was observed that the intensity of the absorption and the appearance of the spectrum in the first nanoseconds after the laser pulse were the same under aerobic and anaerobic conditions. However the decay became much faster when oxygen was present.

Experiments with solutions of 3-methyllumiflavin in polymethylmethacrylate

The shape of the transient absorption difference spectra of 3-methyllumiflavin in methacrylate was the same as the one obtained in benzene solutions, which in turn was similar to the spectra in aqueous buffered solutions at pH > 5·6, except that the lifetime for exponential decay is much longer (about 125 ms at room temperature) (Fig. 4). As shown in Table 1, the lifetimes of transient II and III are exactly the same.

The phosphorescence and the E-type delayed fluorescence spectra obtained from the methacrylate sample were the same as reported by Sun *et al.* (1972).

The phosphorescence lifetime obtained from the

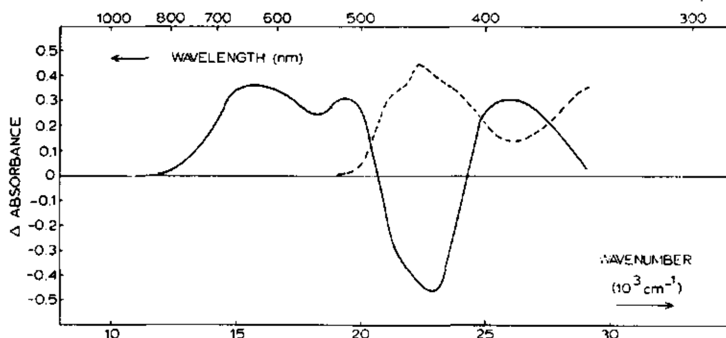

Figure 4. The transient absorption difference spectrum of 3-methyllumiflavin in polymethylmethacrylate at 6μ s after the flash as obtained by flash photolysis. ..... absorption spectrum of a 2 cm disk before flashing; ——— absorption difference spectrum 6 μ s after flashing.

emission at 16,500 cm^{-1} (605 nm) after excitation at 22·200 cm^{-1} (450 nm) was 126 ms. Thus, within experimental error, the decay times of transients II and III are identical to the phosphorescence lifetime.

Transient spectra of other flavins

To test the consistency of the results, other flavins were also investigated at pH 3·0 and at pH 7·0. Lumiflavin, tetraacetylriboflavin and FMN, which all have the same stationary absorption spectra as 3-methyllumiflavin, showed identical transient spectra at pH 7·0 as well as at pH 3·0. The transient spectra obtained with 7,8-dichloro-3-methyllumiflavin, 7-methyl-8-chlorolumiflavin and 7-chloro-8-methyllumiflavin, whose absorption spectra are only slightly different from that of 3-methyllumiflavin, were similar to the transient spectra of 3-methyllumiflavin at about pH 6·0. However, the transient spectra of the first two compounds were not affected by a change in pH, while the transient spectrum of the other flavin derivative showed a distinct dependence on the pH, as was observed with 3-methyllumiflavin.

DISCUSSION

From the foregoing results on the time-resolved transient absorption spectra and from their pH dependence, it follows that three different transients can be observed upon excitation.

The appearance of transient I, with its main absorption in the region from 28,000 to 30,000 cm^{-1} (360–330 nm) and in the region below 14,000 cm^{-1} (710 nm) is neither affected by a change in pH nor by a change in solvent. Since its decay is very short and comparable to the fluorescence decay rate, this absorption may be ascribed to a residual absorption

Table 1. Decay times of the transient absorptions at different wavelengths of 22 μ*M* 3-methyllumiflavin in polymethylmethacrylate as obtained by flash photolysis

Wavelength (nm)	Wavenumber (cm^{-1})	Decay time (ms)
650	15,500	120
530	17,900	124
440	22,700	124
390	25,600	124
364	27,500	128

of 3-methyllumiflavin in its first excited singlet state.

The transient absorption in neutral solution as shown in Fig. 1a after 50 ns and longer, has previously been ascribed to absorption of the flavin in its lowest triplet state (Knowles and Roe, 1968). However, the experiments at lower pH values clearly show that at least two different absorbing species (II and III) are present. II can be ascribed to triplet-triplet absorption of the flavin.

The initial rapid decay of III is only found in fluid solution of pH values $<5·6$. The decay in the buffered solution is exponential, therefore this behaviour can be assigned to the establishment of an equilibrium $III + H^+ \rightleftarrows (III - H^+)$, which at pH = 4·4 is completed after about 120 ns. Moreover the sharp break in the decay time by a few orders of magnitude by changing the pH from 5·6 to 4·4 suggests a pK of about 5 for species III.

Although II and III in fluid solutions of low pH appear as different species, their decay rates in neutral aqueous solution and in the methacrylate sample are the same. Moreover their decay rate is the same as the phosphorescence decay rate. This means that, although the electronic absorption of III is independent of that of II, the mere existence

of II and III is not independent and is connected to the lifetime of the triplet state, as indicated from the phosphorescence measurements.

It is difficult to imagine how one can create two coupled, although independently absorbing, electronic systems from a single flavin molecule. On the other hand, there are indications from optical absorption spectra in aqueous solutions that flavin molecules in the ground state are weakly interacting at concentrations as low as 10^{-4} to 10^{-5} *M* (Müller *et al.*, 1973). Furthermore Song *et al.* found red shifts in the phosphorescence spectra in the same concentration range even in apolar solvents, which they assigned to the formation of dimers of flavin molecules in the ground state (Song *et al.*, 1972). Thus at a concentration of 10^{-4} *M* 3-methyllumiflavin the presence of dimers is to be expected. At higher flavin concentrations, it was demonstrated with nuclear magnetic resonance for FMN that the two flavins are imperfectly stacked face-to-face through the aromatic parts, with the polar side chains opposing each other (Sarma *et al.*, 1968).

Now, when the dimer is excited some reaction between the two flavin molecules in the dimer may occur and some kind of covalently bound intermediate may be formed. The electronic properties of one of the flavin molecules are drastically changed, giving rise to a new absorption band (III), while the π-electronic system of the other flavin molecule is not affected and is left in the triplet state, giving rise to triplet–triplet absorption spectra (II). Such a compound may be stable only in the excited state.

In this way one could explain in principle that (i) in the excited state one part can be protonated without affecting the optical absorption of the other part, and (ii) when the triplet part decays, the other part, protonated or not, also decays with the same rate since the compound is no longer stable when the other part (in the triplet state) decays independently.

Dimerisation in the ground state is to be preferred above excimer formation after excitation, since the transient absorption spectra were already present directly after the laser pulse, while diffusion-controlled excimer formation, even at concentrations of 10^{-3} *M*, is complete after about 70 ns (Post *et al.*, 1971). In our case excimer formation can be excluded since no growing in but only decay was observed on such a time scale.

Before discussing the specific structure of the intermediate compound formed from the flavin dimer after excitation, we first want to mention some striking similarities with the photo-addition reaction of phenylacetic acid and flavin (Brüstlein *et al.*, 1971; Haas and Hemmerich, 1972). First, there are strong indications for the formation of a complex between 'substrate' and flavin in the ground state. In the second place, it was possible to isolate under certain conditions a primary photoproduct in which a benzylgroup was added at the 8-position of the flavin. This species possesses a rather acidic proton at N(1), exhibiting a p*K*-value of about 5. In an earlier paper, Hemmerich *et al.* have already demonstrated the weak acidity and the reactivity of the 8-methyl group of the flavin (Hemmerich *et al.*, 1959). Our experiments with other flavin derivatives showed that the presence of a methyl group in position 8 of the isoalloxazine nucleus was required to generate the pH dependence of III.

Based on our experimental data we suggest that, immediately after excitation of the flavin dimer in neutral aqueous solutions, an intermediate is formed for which we propose the structure given in Fig. 5A. Flavin molecule A is bound to position 8 of flavin molecule B with a simultaneous electron transfer to B and concomitant ejection of a proton from the methyl group at position 8 of flavin molecule A. In acid solution part B of this primary product is protonated. Although the results of our experiments can be better explained by taking into account the presence of dimers, an alternative explanation cannot be excluded (Fig. 5B). This tautomeric structure was proposed to be present in small amount in equilibrium with normal 3-methyllumiflavin in the ground state in order to explain the observation that the protons of the 8-methyl group can be fully exchanged against deuterium under mild conditions (Bullock and Jardetzky, 1965).

One might assume that the equilibrium is displaced towards B when a strong perturbation like an intense light pulse is applied, resulting in a very fast intramolecular proton transfer towards N(1). This compound might absorb light at about 26,000 cm^{-1} and could be expected to exhibit a p*K* value of about 5. This species might also be generated in apolar solvents. Unfortunately, because of its instability, a compound with a structure like Fig. 5B has never been detected, but has been found with pteridins (Pfleiderer *et al.*).

It should be noted that the absorption of transient species III and a part of that of II lie in the same spectral region as the strong absorption of the flavin in the ground state. This makes it impossible to determine quantitatively the contribution of III to

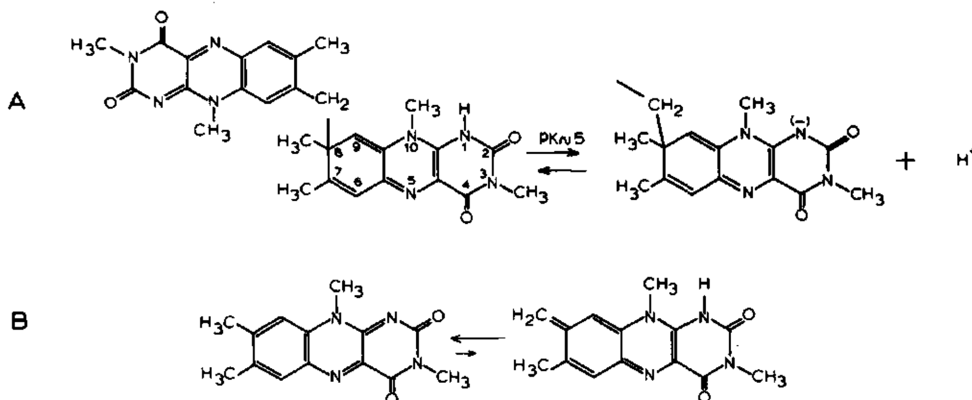

Figure 5. A: Proposed unfolded structure of the intermediate compound formed after excitation of a flavin dimer. B: Alternative tautomeric structure of 3-methyllumiflavin.

the observed changes of absorption. From an experimental point of view the overlap of the different absorptions restricts severely the concentration range (roughly 50–200 μM) and the laser energy ($>$ 5 mJ) that can be applied for the detection of transient absorption III. For these reasons a clear-cut concentration dependence of the appearance of transient III at neutral pH, which might prove the proposed intermediate structure (Fig. 5A), could not be established.

Because of the interest in photochemical reactions of flavins, we hope that this study on a time scale of nanoseconds will contribute to the understanding of these reactions as concerns the pH and time dependence of the transients obtained by excitation of the flavin molecule.

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TIME RESOLVED FLUORESCENCE OF FLAVIN ADENINE DINUCLEOTIDE

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1. Introduction

From a comparison of the quantum efficiencies and lifetimes of the isoalloxazine fluorescence in flavin adenine dinucleotide (FAD) and flavin mononucleotide (FMN) and from the slightly different extinction coefficients of both compounds Spencer and Weber have calculated and approximated the rate constants which describes the strong intramolecular quenching in FAD [1].

One of the assumptions in their derivation is that the observed fluorescence is due to the open or unstacked form of FAD, while the closed or stacked structures have no appreciable fluorescence. These assumptions are among others based on the experimental fact that the shape of the fluorescence spectra of FAD and FMN is almost identical. However, one can also approach this point from another direction. Suppose that the more complete kinetic scheme can be applied for FAD (fig. 1).

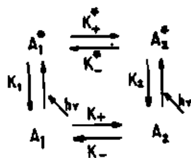

Fig. 1. Kinetic scheme describing the folding and unfolding of FAD in the ground-state and in the excited state.

If both folded and unfolded forms are fluorescent and the emission spectra of both species are overlapping, one should expect that the time course of the fluorescence intensity ($I(t)$) at a given wavelength of emission, after a pulse excitation can be built up by a sum of two exponentials with different amplitude factors:

$$I(t, \lambda) = A'_1 (\lambda) \exp(-t/\tau'_1) + A'_2 (\lambda) \exp(-t/\tau'_2)$$

The derivation of this equation and the expressions of the parameters $A'_1 (\lambda)$, $A'_2 (\lambda)$, τ'_1, τ'_2 are given in a previous work [2]. These parameters are functions of k_+^*, k_-^*, k_1, k_2 and K, where k_+^* and k_-^* are the rate constants in sec^{-1} for folding and unfolding in the excited state, $k_1 = 1/\tau_1$ and $k_2 = 1/\tau_2$ are the rate constants in sec^{-1} for fluorescence and radiationless transitions for the isolated forms and K is the association constant in the ground state, index 1 and 2 refer to the open and closed conformations respectively (see fig. 1).

Furthermore, $A'_1 (\lambda)$ and $A'_2 (\lambda)$ are functions of the emission wavelength λ.

On the other hand, when there is only one fluorescent form, it is expected that the exponential decay is characterized by one time constant and is independent of the emission wavelength. If the unfolded form is fluorescent, the fluorescence lifetime is then given by $\tau'_1 = 1/(k_+^* + k_1)$.

We investigated the time resolved emission of FAD to detect whether or not there was a heterogeneous character of the fluorescence which might be a direct experimental evidence for the existence of more than one fluorescent species. For this study, the single photon counting method was used, which is superior to the phase and modulation fluorometer method [3] because it is more sensitive with respect to the detection of a heterogeneous character of the fluorescence decay. Especially when measurements are made on samples with low quantum yields of the emission, as is the case with FAD (in the order of 0.03 at neutral pH and at room temperature [1], the single photon counting method provides a reasonable resolution of the fluorescence in time and in wavelength.

2. Materials and methods

FMN and FAD (grade III) were obtained from the Sigma Company (St. Louis, USA). FAD was purified with chromatography on DEAE-cellulose (Whatman DE 52) [4]. 0.05 M sodium phosphate buffer at pH 6.9 made from doubly distilled water was used as solvent. The chromophore concentration was always 20 μM.

Fluorescence decays have been obtained with a single photoelectron counting apparatus which has been described earlier [5]. We used a free running flash lamp operating in nitrogen at high pressure. The exciting wavelength and the emission wavelength are selected by grating monochromators. The wavelength band were 10 nm and 6 nm for excitation and emission respectively. The response function $g(t)$ of the apparatus, which is used for the decay analysis, was obtained with a reference compound by a method described elsewhere [6]. The reference used here was 1,1,4,4 tetraphenyl 1–3 butadiene, (TPB) obtained from Koch Light. Its time constant is 1.78 nsec [7]. Analysis of the data has been performed with a computer program based on the method of modulatrix functions [8]. The program was designated to determine the three parameters relative to decays which are a sum of two exponential terms according to the following expression:

$$I(t) = [C_1\, e^{-t/\tau_1} + (1-C_1)\, e^{-t/\tau_2}]\, A \qquad (1)$$

where A is a factor of normalization. The fit of these parameters was checked by calculating the convolution of the decay (1) with $g(t)$. This computed curve was then compared visually with the experimental curve and the weighted residual was calculated [9].

The average time constant $<\tau>$ was also calculated. It is defined by the following expression:

$$<\tau> = \frac{M_1\,[i(t)]}{M_0\,[i(t)]} - \frac{M_1\,[g(t)]}{M_0\,[g(t)]}$$

$M_1\,[i(t)]$ and $M_0\,[i(t)]$ designate the moments of order one and of order zero of an experimental decay $i(t)$, $M_1\,[g(t)]$ and $M_0\,[g(t)]$ the moments of the response function $g(t)$.

3. Results and discussion

In table 1 the results for the FMN and FAD fluorescence kinetics at variable emission wavelengths, constant excitation wavelength (370 nm) and constant temperature (20°C) are collected. A computer program was used in which the experimental data were analyzed into two time constants with their relative amplitudes (see Materials and methods). In general the weighted residual showed no deviation from its statistical value so that there was good agreement between experimental and convoluted curves. In table 1 only the lifetimes (τ_1) with the largest weight factor C_1 and the average lifetime $<\tau>$ are included. The data obtained with FMN are in good agreement with the results of Spencer et al. [10]. Only one lifetime is observed and the lifetime is constant across the fluorescence band. The same tendency can be obser-

Table 1
Two component analysis of the FAD and FMN fluorescence at different emission wavelengths

Fluorescence wavelength (nm)	FAD			FMN		
	τ_1 (nsec)	C_1	$<\tau>$ (nsec)	τ_1 (nsec)	C_1	$<\tau>$ (nsec)
500	2.72	0.98	2.93	4.55	1.00	4.55
520	2.82	0.95	2.79	4.70	1.00	4.67
560	2.68	0.99	2.84	4.69	1.00	4.58

Excitation wavelength 370 nm, temperature 20°C.

ved with FAD, where the data (table 1) point to one single lifetime, which is within 0.2 nsec constant across the fluorescence band. It was not possible to analyse accurately the fluorescence at wavelengths longer than 560 nm because the reference compound (TPB) did not emit at these wavelengths. The lifetimes of the second component, which is present in very low percentage (<5%), were always longer than about 5 nsec and the variation between them is very large. Although impurities cannot be excluded, when weak emissions are detected, it might be possible that the appearance of the small percentage of the second component is a result of the calculation method employed especially in cases where the main component has a short lifetime. As a test for the significance of our results some experimental data were analysed into one single lifetime and in these cases the lifetimes are within 0.1 nsec identical with τ_1. Also the temperature dependence of the fluorescence lifetime at constant emission wavelength (520 nm) was studied. From the data in table 2 it is obvious that especially at the lower temperatures the fluorescence is characterized by one single exponential decay. For FAD the lifetime decreases with increasing temperature from 3.5 nsec at 2°C to 2.1 nsec at 34°C. The large variation of the fluorescence lifetime with temperature illustrates the strong dynamic character of the fluorescence quenching. For FMN the variation is within a much smaller range namely 4.9 nsec at 2°C to 4.5 nsec at 34°C. The same tendency is observed by Spencer and Weber [1]. However our lifetimes of the FAD fluorescence seem to be about 0.2–0.3 nsec longer. The single exponential decay of the FAD fluorescence justifies the assump-

Table 2
Two component analysis of the FAD and FMN fluorescence at different temperatures

Temperature (°C)	FAD			FMN		
	τ_1 (nsec)	C_1	$<\tau>$ (nsec)	τ_1 (nsec)	C_1	$<\tau>$ (nsec)
2	3.51	0.99	3.69	4.87	1.00	4.79
12	3.11	1.00	3.20	–	–	–
20	2.82	0.95	2.79	4.70	1.00	4.67
27	2.47	0.91	2.76	4.59	1.00	4.55
34	2.07	0.94	2.36	4.52	1.00	4.50

Excitation wavelength 370 nm, emission wavelength 520 nm.

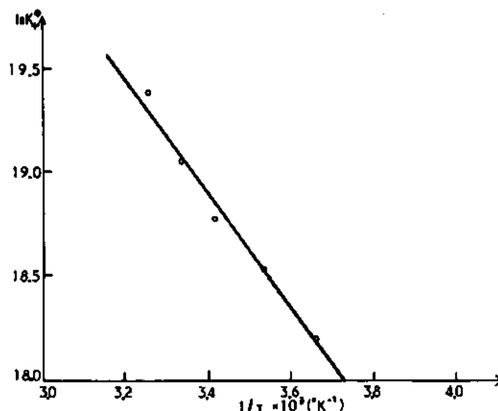

Fig. 2. Plot of $\ln k_+^*$ vs $1/T$. k_+^* can be assumed as the rate constant (in sec^{-1}) of formation of the complex between the excited isoalloxazine and adenine moieties. T = temperature in °K.

tions that $1/\tau_2' >> 1/\tau_1'$ and that $1/\tau_1' \approx k_+^* + k_1$ (fig. 1). In the complex, extra radiationless processes are present as compared to the open form. Radiationless deactivation is so rapid that $k_-^* << k_2$ and A_1^* is probably created only by direct excitation. As pointed out earlier, because the stacked form is nonfluorescent the only rate parameter that can be evaluated is k_+^* by taking the observed lifetime of the FAD fluorescence as τ_1' and the observed lifetime of the FMN fluorescence as $1/k_1$ [1]. The k_1^* values at different temperatures are shown in the form of an Arrhenius plot (fig. 2). From the slope of the plot the activation energy for complex formation in the excited state is calculated as 5.45 Kcal/mole. This value is in the same order of magnitude as found by Spencer and Weber [1]. We can conclude from these results, that the fluorescence of FAD in aqueous solution arises from one single species, which corresponds to an aqueous environment of the isoalloxazine moiety.

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Intrinsic Luminescence Studies on the Apoenzyme and Holoenzyme of Lipoamide Dehydrogenase

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The following fluorescence parameters have been investigated: emission and excitation spectra, relative quantum yields, polarization values and lifetimes of the apo- and holoenzyme of lipoamide dehydrogenase. Furthermore, fluorescence perturbation techniques have been used to characterize qualitatively the tryptophan and flavin environment in the protein. Also emission spectra at low temperature have been recorded to examine changes occurring on strong immobilization of the enzyme. From lifetimes and quantum yields of the protein fluorescence it can be concluded that the molecular structures of the two apoenzymes, which can be prepared from this enzyme, are different. The quenched protein fluorescence of the holoenzyme is characterized by a very short fluorescence lifetime due to radiationless energy transfer from tryptophan to flavin. Excitation polarization spectra of flavin and tryptophan fluorescences show that the chromophores are rigidly bound to the protein. Luminescence studies at liquid nitrogen temperatures reveal that the triplet state of the tryptophanyl-residues is not involved in energy transfer to the flavin.

Collisional fluorescence quenching with KI shows in the dimeric holoenzyme, in contrast with the free molecules, only a small reduction in fluorescence intensity of tryptophanyl and flavin residues. Charge effects might be responsible for these observations. It can be derived from the experimental results together with the indication that only one of the two tryptophans is transferring its excitation energy to the flavin, that the distance between donor and acceptor is 1.3–1.6 nm.

Lipoamide dehydrogenase from pig heart shows in contrast to many other flavoproteins a rather high fluorescence yield of the flavin, which makes it especially suitable for studying different fluorescence parameters. In addition another fluorogenic chromophore in this enzyme is tryptophan. The enzyme has a dimeric structure and contains four tryptophans and two flavins (as FAD) per holoenzyme [1]. Fluorescence and other spectroscopic techniques have been used to study the large structural differences between apoenzyme and holoenzyme and changes induced in the latter by the employment of perturbation techniques [2,3]. Thus the protein fluorescence of the apoenzyme is enhanced when compared with that of the holoenzyme [4]. In addition, the tryptophan fluorescence emission maximum of the protein is shifted hypochromically as compared to that of free tryptophan suggesting that the tryptophan residues are located in an apolar environment [4]. Radiationless energy transfer from the tryptophans to the flavin has been reported [3,5].

In this paper the emission properties of lipoamide dehydrogenase have been reinvestigated in more detail in order to obtain a better insight into the physical processes underlying these phenomena.

MATERIALS AND METHODS

Preparation of Enzyme Samples

The preparation of lipoamide dehydrogenase has been described previously [6]. The apoenzyme was prepared in two different ways. Apoenzyme I was obtained according to Kalse and Veeger [2]. Apoenzyme II was prepared with guanidine-HCl (procedure 2), as described by Brady and Beichok [7]. Both apoenzymes are able to recombine with FAD to active holoenzyme. Apoenzyme I has a monomeric structure and its CD spectrum in the 260 to 300-nm region resembles that of the holoenzyme [4]. On the other hand the conformation of apoenzyme II differs

Abbreviation. CD, circular dichroism.
Enzyme. Lipoamide dehydrogenase or NADH:lipoamide oxidoreductase (EC 1.6.4.3).

from that of the holoenzyme [7], but its structure seems to be dimeric (Massey, V., personal communication). Because of their instability the apoenzymes were stored on ice and used directly after preparation. Most experiments were conducted at 4 °C unless otherwise stated. The protein concentrations used in the measurements varied from 0.5–2 mg/ml, which is low enough to eliminate inner filter and reabsorption effects in fluorescence studies.

Phosphate-buffered solutions of pH 7.2 containing 0.3 mM EDTA were used in the experiments. All other chemicals used were of the highest purity available.

The incorporation of 3-methyllumiflavin into polymethylmethacrylate was performed as described in the literature [9].

Absorption Spectra

Absorption spectra were measured on a Cary 14 spectrophotometer equipped with thermostated cell-compartments.

Measurements of Emission Parameters

The fluorescence spectra were recorded in the ratio mode on a Hitachi-Perkin Elmer MPF-2A spectrofluorometer equipped with a thermostated cell holder. For the determinations of quantum yields the fluorescence spectra were corrected for the nonlinearity of the detection system, for which a calibration curve was obtained with a standard lamp [10]. The corrected emission spectra were plotted on a wavenumber scale and the area under the curve was determined by numerical integration using a standard computer program. This integral was compared with one obtained from a L-tryptophan solution in the same phosphate buffer at pH 7.2 exhibiting the same absorbance at the excitation wavelength (295 nm). In this way fluorescence yields were obtained relative to a value of 0.14 for L-tryptophan [11]. Fully corrected excitation spectra were recorded as described by Chen [12] using 5 g/l rhodamine B in ethylene glycol as quantum counter.

Fluorescence polarization values were obtained with an apparatus based on the design of Weber and Bablouzian [13]. The excitation light from a stabilized 150-W Xe-source was passed through a Bausch and Lomb grating monochromator (1200 lines/mm, blazed at 300 nm). Band-pass and cut-off filters (Schott Jena UG 14 and BG 305) were used for isolating the protein fluorescence and cut-off filters (Schott Jena OG 530) for observing the flavin fluorescence. The degree of polarization could be directly read off from a DANA model 5403 digital voltmeter. Depending on the signal intensity the absolute accuracy varied between 0.005 and 0.01. Fluorescence lifetimes were measured with a 28-MHz phasefluorometer [14]. The same filters as above were used for observation of the emissions. The accuracy of the measurements is within 0.2 ns. Some filters introduced a weak background fluorescence, which has to be taken into account especially in the case of weak emissions.

Emission spectra at 80 K were recorded on a very sensitive phosphofluorometer [15]. In order to obtain a rigid glass at low temperature, concentrated protein solutions were diluted with a buffered 30% sucrose solution of pH 7.2. The measurements were performed in a small flat quartz cell with 0.1-mm light path. The cell was inclined at an angle of 20° to the incident beam and frontal observation of the emission was employed to overcome reabsorption of emitted light. After measuring a spectrum at 277 K, the sample was gradually cooled to 80 K with a cold nitrogen flow and recorded again. The long-lived emission was isolated from the direct fluorescence by rotating the choppers in the excitation and emission light paths 180° out of phase.

Two methods for recording the phosphorescence lifetimes were employed. In general long and moderately strong delayed emission lifetimes were measured with a shutter-fast recorder (Philips) system. Fast and weak signals were fed directly in a Varian C-1024 time averaging computer, which was read out after accumulation of many transient signals.

RESULTS AND DISCUSSION

Fluorescence Properties of Apo- and Holoenzyme of Lipoamide Dehydrogenase

The fluorescence emission spectra of the apoenzyme I and II, the holoenzyme and L-tryptophan at 277 K are shown in Fig. 1. The excitation wavelengths employed were 280 nm (Fig. 1 A) and 295 nm (Fig. 1 B). Light of 295 nm excites almost exclusively the tryptophan residues of the protein. Therefore, this excitation wavelength was used in studying energy transfer from tryptophan to flavin (*cf.* below). The emission maxima of the tryptophan residues in the three proteins, located at about 325 nm, exhibit a blue shift as compared to free tryptophan. This indicates that the tryptophans are located in an apolar environment [16]. The fact that the position of the maximum of the tryptophan fluorescence of the holo- and apoenzymes is practically the same suggests that binding of FAD to the apoproteins has little influence on the direct environment of the tryptophan residues. The intensity of the short wavelength region of the emission band increases, when the samples are excited at shorter wavelength than 295 nm. The positions of the maxima of apoenzyme I and holoenzyme shift hypochromically indicating that tyrosyl residues have a larger contribution to the fluorescence of these proteins than to that of apoenzyme II. In this context it should be

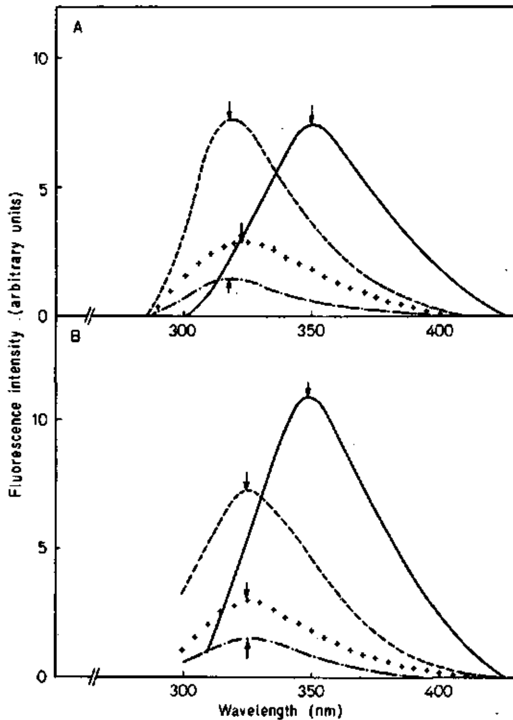

Fig. 1. ***Fluorescence emission spectra of holo- and apolipoamide dehydrogenases and of free tryptophan.*** (A) Excited at 280 nm; (B) excited at 295 nm. The absorbance at 280 nm for all four solutions was 0.35. (----) Apoenzyme I; (++++) apoenzyme II; (-·-·-) holoenzyme; (——) tryptophan. The pH was 7.2 for all samples. The temperature was 4 °C. The arrows indicate the maxima of the protein fluorescence spectra

noted that the protein contains 7 tyrosyl residues per flavin. Apart from the quantum yield, which is much lower in the holoenzyme, the emission spectra of holoenzyme and apoenzyme I are rather similar, a result which could be expected considering the rather similar C.D. spectra of the two proteins [4].

In Fig. 2A the polarization values of the fluorescence of the tryptophan residues of the three proteins have been plotted against the excitation wavelength. The values obtained for the holoenzyme were subject to larger errors because of the quenched protein fluorescence and the resulting less favourable signal to noise ratio. The degree of polarization of the indole part of tryptophan is intrinsically low because of the presence of more than one electronic transition, which overlap at the different excitation wavelengths [17]. In all three cases almost maximum polarization values of the tryptophan residues of the different proteins are observed. This indicates that the motion of the tryptophan residues is restricted in the nanosecond region and that energy transfer between the tryptophans themselves, which causes depolarization of the fluorescence, hardly occurs.

Also shown in Fig. 2B are the polarization spectra of the flavin fluorescence of the holoenzyme and of the flavin emission of 3-methyllumiflavin in polymethyl-methacrylate. For comparison the absorption spectra are also shown. The degree of polarization is very high (> 0.4) when excited at the red side of the first absorption band of the flavin. This demonstrates that the flavin is rigidly bound to the protein. On the blue side of the absorption maxima of both flavin spectra a small but consistent depolar-

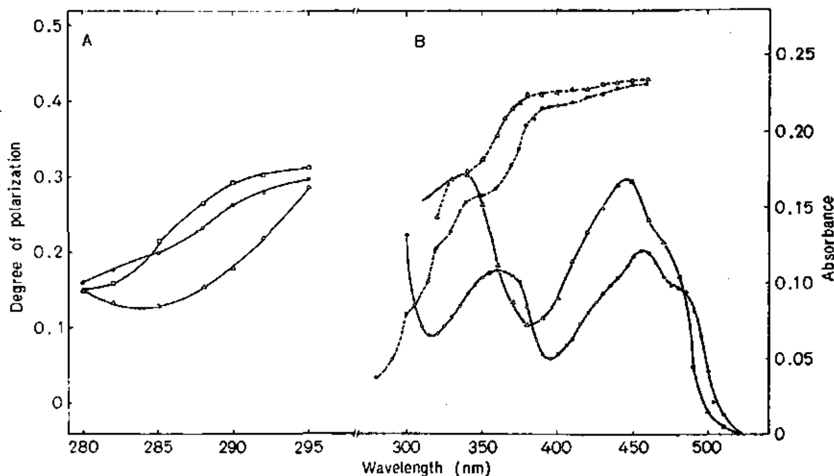

Fig. 2. ***Fluorescence polarization spectra of holo- and apolipoamide dehydrogenase.*** (A) The degree of polarization of the protein fluorescence as a function of the excitation wavelength: (□——□) apoenzyme I; (○——○) apoenzyme II; (△——△) holoenzyme. (B) The degree of polarization of the flavin fluorescence (dashed line) of the holoenzyme and of 3-methyllumiflavin in polymethylmethacrylate as a function of the excitation wavelength. The absorption spectra (solid line) of both samples are also included: (○——○) holoenzyme; (△----△) 3-methyllumiflavin in plastic. Spectral bandwidth, 5 nm. The temperature was 4 °C

ization can be observed coinciding roughly with the shoulder on the blue side of the absorption maximum, which is attributed to vibrational fine structure. It is not possible to give a correct explanation for this small effect. Upon excitation at shorter wavelengths a sharp transition is observed. The degree of polarization decreases rapidly between 400 and 370 nm and a shoulder in the 360-nm region is observed, which coincides with the absorbance maximum. Interesting is the large difference in polarization at the maxima of the second absorption bands of the model flavin and the enzyme-flavin. An influence of the N-3 methyl-group of the model flavin on the direction of the second electronic transition moment with respect to the first, might be responsible for this difference. On the other hand distortion of the FAD upon binding to the protein could lead to a different direction of the transition moment. It can be calculated from the polarization values [18] that the angle between the S_1- and S_2-excitation vectors is 26–27° in 3-methyllumiflavin and 30–31° in the enzyme-bound FAD. In the holoenzyme a further gradual decrease in the degree of polarization is observed at a still shorter wavelength. At 295 nm, where the flavin fluorescence is mainly sensitized, the degree of polarization is low, but is still positive. This sensitized flavin fluorescence is most clearly demonstrated with the corrected flavin fluorescence excitation spectrum of the holoenzyme when it is compared with its light absorption spectrum and the excitation spectrum of FAD. At wavelengths shorter than 295 nm the excitation spectrum of the enzyme exhibits an intensity which lies in between both other spectra. This points to a rather efficient energy transfer from tryptophan to flavin. The polarization values of the sensitized and direct emissions can yield some information concerning the question whether or not the tryptophans are randomly oriented in the protein with respect to the flavin. For a similar case the following expression was used [19]:

$$\frac{1}{P_{DA}} - \frac{1}{3} \simeq \frac{6}{5}\left(\frac{1}{P_D} - \frac{1}{3}\right)\left(\frac{1}{P_A} - \frac{1}{3}\right). \quad (1)$$

P_{DA} is the observed sensitized polarization of the fluorescence when the donor is excited selectively, P_D is the limiting polarization of the donor fluorescence excited at the same wavelength and P_A is the polarization of the acceptor fluorescence excited at the wavelength of the overlap maximum. We measured $P_A = 0.28$ and $P_D = 0.30$. These values give $P_{DA} = 0.08 \pm 0.01$ with the aid of Eqn (1). The measured $P_{DA} = 0.09 \pm 0.01$. Both values are in good agreement suggesting that the assumption of random orientation of the tryptophans with respect to the flavin is valid.

Data concerning the quantum yields and lifetimes of the protein fluorescence for the three cases are collected in Table 1. It should be noted that the extent of fluorescence quenching is larger for apoenzyme II than for apoenzyme I. This is reflected in the quantum yields as well as in the lifetimes. The fluorescence lifetime of apoenzyme II is considerably shorter than that of apoenzyme I. These differences in the various fluorescence parameters reflect the structural differences between the two apoenzymes and are presumably related to subunit-interaction in apoenzyme II. The lowest quantum yield and shortest lifetime are observed in the holoenzyme, which must be partly due to radiationless transfer of excitation energy to the flavin.

Table 1. *Tryptophan fluorescence lifetimes* (τ) *and quantum yields* (Q) *of holo- and apolipoamide dehydrogenases*
The experimental conditions are as described in Materials and Methods. Excitation at 295 nm. Quantum yields were obtained by comparison with the quantum yield of free tryptophan [11]

Enzyme	τ	Q
	ns	
Apoenzyme I	3.8	0.09
Apoenzyme II	2.4	0.03
Holoenzyme	1.6	0.01

Luminescence at Low Temperature

Total and delayed emission spectra obtained by excitation at 290 nm are shown in Fig. 3. The characteristic fine structure of the tryptophan phosphorescence [20] is also observed in holo- and apoenzymes (see Fig. 3B). From these data it is evident that no tryptophan triplet excitation energy is transferred to the flavin in the holoenzyme. Only a small amount of delayed flavin fluorescence was observed, eliminating tryptophan triplet-flavin singlet transfer [21]. Bearing in mind that the transition moment from the triplet to the ground state of tryptophan points perpendicular to the indole plane and that the fluorescence transition vector lies in the plane, one can assume that the mutual orientation of both chromophores is unfavourable for triplet-singlet energy transfer [22]. Similar observations were made by Risler for L-lactate dehydrogenase from yeast [23]. From this it might be concluded that the flavin and indole moieties are more or less oriented in the same plane.

Since no sensitized phosphorescence of the flavin (maximum at 610 nm) could be detected by excitation at 290 nm, triplet-triplet energy transfer can be excluded. However flavin phosphorescence (lifetime 420 ms) can be easily detected by excitation at 450 nm. Triplet-triplet energy transfer requires a close contact between the chromophores [24]. Our phosphorescence data do not indicate such an interaction, therefore the distance between tryptophans

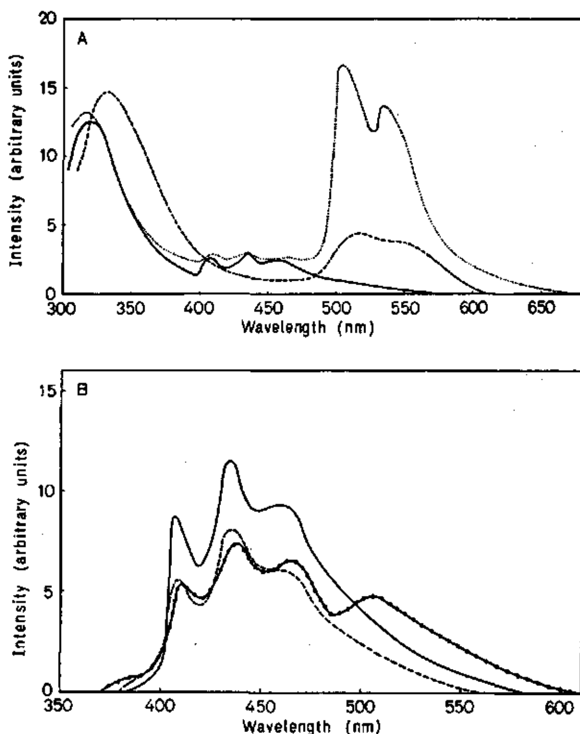

Fig. 3. *Total (A) and delayed (B) emission spectra of apo- and hololipoamide dehydrogenase (uncorrected).* Excitation at 290 nm. Conditions as mentioned in Materials and Methods. (A) (– – – –) Holoenzyme at 293 K; (······) holoenzyme at 80 K; (——) apoenzyme I at 80 K. (B) (–·–·–) Holoenzyme at 80 K; (——) apoenzyme I at 80 K; (– – – –) tryptophan (10 μM) at 80 K. The concentration of all protein solutions was 1 mg/ml. The gain in B is 6 times that in A; no corrections were applied for different chopping modes

and flavins is probably larger than their collisional diameters.

The phosphorescence lifetimes of tryptophan in holo- and apoenzyme I are of the same order of magnitude, 2.0 and 2.6 s respectively. This shortened phosphorescence decay as compared with the phosphorescence lifetime of free tryptophan at pH 7.2 (5.0 s) suggests a direct interaction with other amino acid residues which deactivate the triplet state of a neighbouring tryptophan. Song and Kurtin [17] have shown that the phosphorescence lifetime of indole is strongly dependant on substituents in the nucleus.

Upon cooling the holoenzyme solution to 80 K both flavin and tryptophan fluorescences were blue shifted. The flavin fluorescence maximum is shifted from 520 nm at 293 K to 505 nm at 80 K. The fluorescence intensity is increased and the vibrational fine structure is better resolved. Solvent relaxation processes around the excited flavin might therefore be important in the holoenzyme. These phenomena will be investigated in more detail with flavin models.

Collisional Quenching with Potassium Iodide (KI)

A titration with KI [25,26] was performed monitoring the decrease in fluorescence intensity of flavin and tryptophan in the holoenzyme. In Fig. 4A the fractional fluorescence of the flavin in the holoenzyme as a function of the KI concentration [27] is shown in comparison with FMN and FAD in buffered solution of pH 7.2. The data for free and bound tryptophan are given in Fig. 4B. Also the quenching behaviour at 4 °C of tryptophan and flavin fluorescence in the holoenzyme is represented in Fig. 4. Several remarkable features can be observed from these data. The rate constants for quenching are much smaller for protein-bound flavins and tryptophans than the corresponding rate constants of the free chromophores At 4 °C the protein tryptophan fluorescence is not quenched at all by iodide ions. The enzyme-flavin fluorescence is less quenched at 4 °C than at 20 °C. These results support our previous conclusion that the enzyme exists at different temperatures in different protein conformations in which a tryptophan is more buried at 4 °C than at 20 °C [28]. This is also

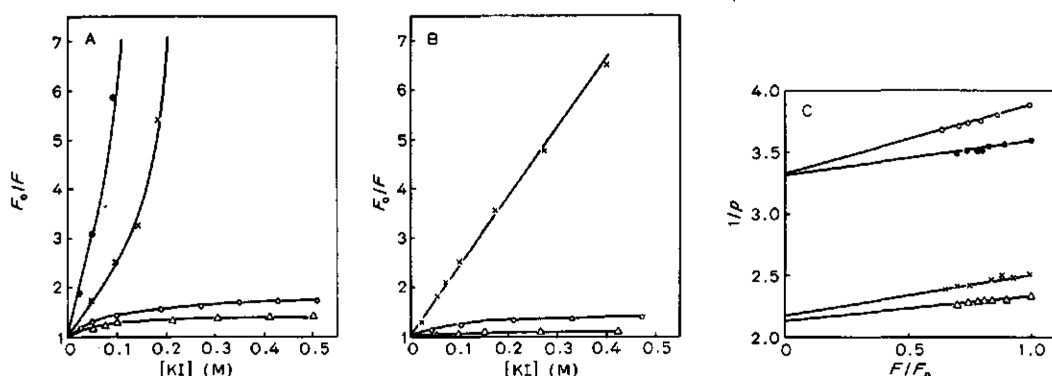

Fig.4. *Stern-Volmer and Perrin plots for the KI quenching of the fluorescence of flavin and tryptophan.* (A) Quenching of the flavin emission (520 nm) upon excitation at 440 nm; (●——●) FMN; (×——×) free FAD; (○——○) enzyme-bound FAD at 20 °C and (△——△) at 4 °C. (B) Quenching of the tryptophan emission, excitation at 295 nm, emission at 335 nm; (×——×) free tryptophan; (○——○) holoenzyme at 20 °C and (△——△) at 4 °C. (C) The reciprocal degree of polarization is plotted against the fractional fluorescence (520 nm) of the flavin in the holoenzyme, the concentration range of added KI was 0–0.1 M, (×——×) excitation at 440 nm, 20 °C and (△——△) at 4 °C; (○——○) excitation at 370 nm; 20 °C, (●——●) at 4 °C. Generally the concentrations were 20 μM. The pH was 7.2

confirmed in plotting the reciprocal polarization ($1/p$) against the fractional flavin fluorescence (F/F_0) of the holoenzyme (Fig.4C), where the first linear part of the KI-titration curve was used. The relationship between $1/p$ and F/F_0 is derived from the classical Perrin equation (Eqn 2), where the fluorescence lifetime τ is substituted by $\tau_0\, F/F_0$ (F_0 and τ_0 are the quantities in the absence of I^--ions) [29].

$$\frac{1}{p} = \frac{1}{p_0} + \left(\frac{1}{p_0} - \frac{1}{3}\right)\frac{\tau}{\varphi}. \tag{2}$$

p_0 is the limiting polarization of the fluorescence, φ is the rotational correlation time of the macromolecule and is equal to $\eta V/kT$, where V is the effective volume of the hydrated sphere, η the viscosity of the solution, T the absolute temperature and k the Boltzmann constant. It is seen that additions of KI up to 0.1 M KI are leading to a linear relationship indicating a collisional quenching process, which shortens the fluorescence lifetime. The different slopes at high and low temperature illustrate the different quenching behaviour of the enzyme at these temperatures.

Another characteristic is the non-linear Stern-Volmer plot of the holoenzyme. At about 0.1 M KI where tryptophan, FMN and FAD show almost linear behaviour a clear deviation from linearity is seen with the enzyme. At still higher concentrations of KI quenching occurs to a minor extent and a residual flavin fluorescence of about 60–70% is left, depending on the temperature of the experiment. In principle several explanations might be given for this heterogeneous fluorescence quenching by KI. The protein structure and therefore the flavin environment might be affected by increasing the ionic strength with KI. We tested this possibility using higher concentrations of KNO_3 instead of KI and found no salt effect on the flavin fluorescence of the holoenzyme.

Addition of a weaker quencher like KBr up to 0.3 M did not affect at all the flavin fluorescence of the holoenzyme, while the fluorescence intensities of the free coenzymes were reduced to 10% for FMN and 16% for FAD. The Br^- ion is smaller than the I^- ion and therefore can reach the flavin easier. The reason that no quenching with KBr and only a small quenching with KI is observed indicates that probably a negative charge is present near the flavin so that repulsive forces completely prevent collisions with Br^-. The larger, more polarizable, I^- ion is less repelled and therefore the flavin fluorescence is affected so some extent from a longer distance. As pointed out by Lehrer [26], Lakowicz and Weber [30] fluorescence quenching by I^- is in contrast with that by O_2 highly dependent on charge effects.

Energy Transfer

Electronic energy transfer from a donor to an acceptor molecule has been treated theoretically by Förster [31] and applications in biological systems have been discussed and reviewed by Eisinger *et al.* [32] and Steinberg [33]. The quantity that can be measured is the transfer efficiency, which for a single donor-acceptor pair is defined as [31]:

$$E = \frac{1}{1 + (r/R_0)^6} \tag{3}$$

where r is the donor-acceptor separation and R_0 the Förster critical distance for 50% efficient energy transfer:

$$R_0 = (8.8 \times 10^{-25}\, \varkappa^2\, Q_D{}^0\, n^{-4}\, J)^{1/6}. \tag{4}$$

In this formula $\varkappa^2$ is the dipole-dipole orientation factor, $Q_D{}^0$ the fluorescence quantum yield of the donor in the absence of energy transfer, n the refractive index of the medium and J the spectral overlap integral:

$$J = \frac{\int_0^\infty F_D(\bar{\nu}) \cdot \varepsilon_A(\bar{\nu})/\bar{\nu}^4 \cdot d\bar{\nu}}{\int_0^\infty F_D(\bar{\nu}) \cdot d\bar{\nu}} \quad (5)$$

in which $\varepsilon_A(\bar{\nu})$ is the molar absorption coefficient of the acceptor and $F_D(\bar{\nu})$ the fluorescence intensity of the donor at wavenumber $\bar{\nu}$. Energy transfer can be measured directly when sensitized emission of the acceptor can be observed. The energy transfer efficiency can also be determined indirectly from the reduction of the quantum yield and lifetime of the donor emission measured in the absence and in the presence of acceptor molecules.

If it is assumed that quenching of the tryptophan fluorescence is mainly due to radiationless energy transfer to the flavin then an estimate of the transfer rate can be obtained from the following equations:

$$1/\tau_D{}^0 = k_f + k_s \text{ and } 1/\tau_D = k_f + k_s + k_{DA} \quad (6)$$

k_f, k_s and k_{DA} are the rate constants respectively for fluorescence, radiationless deactivation and energy transfer from the emitting singlet state. k_{DA} was calculated from the observed fluorescence lifetimes of holoenzyme and apoenzyme and values of $3.6 \times 10^8\ s^{-1}$ and $2.1 \times 10^8\ s^{-1}$ were found using the τ values of apoenzyme I and II, respectively (*cf.* Table 1). These values are larger than the corresponding k_f-values, which are $2.4 \times 10^7\ s^{-1}$ and $1.3 \times 10^7\ s^{-1}$, respectively (calculated from $k_f = Q_f/\tau$ where Q_f = quantum yield in apoenzymes). Obviously, energy transfer is competing with fluorescence.

In Table 2 overlap integrals (J) and R_0 values were calculated for three different tryptophan environments from Eqns (4) and (5) assuming two different average orientation factors, namely $\varkappa^2 = 2/3$, in which the dipoles are rapidly rotating and thus are randomly oriented with respect to each other and $\varkappa^2 = 0.476$, where the dipoles are randomly oriented, but fixed [34]. It is clear that small alterations in orientation and in overlap of absorption and emission bands have a small effect on the critical transfer distance R_0 because of the 6th root dependance of R_0 on the spectral overlap integral (J) and the orientation factor $\varkappa^2$.

Without the knowledge of the amino-acid sequence and/or the three-dimensional structure of the protein it is not possible to determine exact distances and relative orientations for each pair of chromophores from energy transfer experiments alone, because it is impossible to evaluate more than one distance from one measured parameter. Therefore only a crude model can be developed. In the model used it is assumed that the donor molecules are randomly distributed within a sphere of diameter D and the acceptor is located either on the surface of this sphere (Fig. 5, insert A) or separated by a distance of $D/2$ from this surface (Fig. 5, insert B). For simplicity the donor molecules are supposed to transfer independently to the acceptor and transfer between donor molecules is neglected. The latter argument is justified from the polarization values. General formulas given by Badley and Teale [35] are adapted for the average transfer efficiency $\bar{E}$ to give the following equations: For the fluorescence yields:

$$\bar{E}_Q = 1 - \frac{12}{1+2K} \int_K^{1+K} \frac{X^7[X-(K+1)](K-X)}{X^6+(R_0/D)^6} dX \quad (7)$$

and for the fluorescence lifetimes:

$$\bar{E}_\tau = 1 - \frac{12}{(1-\bar{E}_Q)(1+2K)} \int_K^{1+K} \frac{X^{13}[X-(K+1)](K-X)}{[X^6+(R_0/D)^6]^2} dX. \quad (8)$$

In these equations the R_0-values are based on $\varkappa^2 = 2/3$ and K is a parameter which depends on the distance of the acceptor from the sphere (diameter D). In the first case (Fig. 5, insert A) $K = 0$. When the integration is performed for a whole range of R_0/D values, curves I and II of Fig. 5 are produced. In the second case (Fig. 5, insert B) where $K = 0.5$, curves III and IV are generated. From the experiments $\bar{E}$ can be determined from the quantum yields and lifetimes measured for holo- and apoenzymes:

$$\bar{E}_Q = 1 - Q/Q_0 \text{ and } \bar{E}_\tau = 1 - \tau/\tau_0, \quad (9)$$

where Q_0 and τ_0 denote the quantities for the apoenzymes. From the data in Table 1 it can be derived that for apoenzyme I, $\bar{E}_Q = 0.89$ and $\bar{E}_\tau = 0.58$ and for apoenzyme II, $\bar{E}_Q = 0.66$ and $\bar{E}_\tau = 0.33$. If D is assumed to be 8.4 nm (twice the Stokes radius [36]) and using R_0 values taken from Table 2 it is obvious that there is no good agreement between

Table 2. *Spectral overlap integrals* (J) *and critical transfer distances* R_0 *for the tryptophan-flavin system of lipoamide dehydrogenase*

Donor in	$10^{15} \times J$	R_0[a]	R_0[b]
	$M^{-1} \cdot cm^6$	nm	nm
Apoenzyme I	8.1	2.09	1.98
Apoenzyme II	8.2	1.74	1.65
Free tryptophan	11.3	2.40	2.25

[a] R_0 calculated for $\varkappa^2 = 2/3$.
[b] R_0 calculated for $\varkappa^2 = 0.476$ [37].

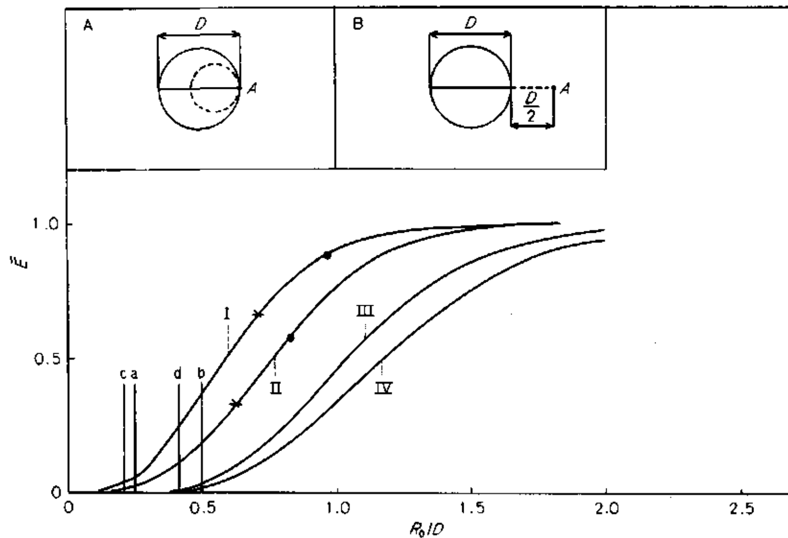

Fig. 5. *Plots* $\bar{E}$ *of the average energy transfer efficiency against* R_0/D. Curves I and II were obtained from Eqns (7) and (8), respectively, by setting $K = 0$ (insert A). Curves III and IV were obtained in the same way by setting $K = 0.5$ (insert B). (●) Experimental transfer efficiencies $\hat{E}$ based on apoenzyme I, (×) based on apoenzyme II. (a) R_0/D calculated for apoenzyme I (*cf.* Table 1) and $D = 4.2$ nm (monomer) (b) for apoenzyme I and $D = 8.4$ nm (dimer) (c), for apoenzyme II and $D = 4.2$ nm (d) for apoenzyme II and $D = 8.4$ nm

the calculated and experimental transfer efficiencies (see Fig. 5). This can be explained by the fact that in the applied model only two tryptophans and one flavin are present per subunit. These two tryptophans are probably distributed within a much smaller volume than the volume occupied by the whole subunit (*cf.* dotted sphere in insert A of Fig. 5) or, alternatively, one tryptophan is located in the neighbourhood of the flavin and transfers very efficiently while the other one is too far removed to transfer to the flavin. If the acceptor is at a certain distance $D/2$ from the surface (Fig. 5, insert B) the discrepancy is even more evident. Assuming both tryptophans contributing to the energy transfer in a random distribution according to model A of Fig. 5, one can calculate the diameter of the sphere in which the three components are located. Depending on the apoenzyme taken as reference for the calculation (apoenzyme I has a higher quantum yield and a longer lifetime than the apoenzyme II), the diameter is 1.3—2.0 nm. The latter value is more probable since it is based on the values obtained from apoenzyme I, which in contrast to apoenzyme II recombines spontaneously with FAD [3]. If this mechanism is operating it is clear that the energy transfer takes place within the subunits (Stokes radius of holoenzyme = 4.2 nm [36]). However, because of the very poor fit between calculated and experimental efficiencies, the hypothesis that the tryptophans are randomly oriented with respect to the flavin is doubtful.

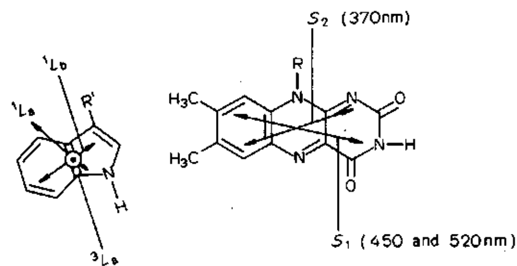

Fig. 6. *Relative parallel orientation of the indole and isoalloxazine planes if it is assumed that one tryptophan molecule participates in energy transfer.* The angle between the emission transition vector of the flavin (S_1) and the absorption transition vector of tryptophan (probably ^1L_a) is approximately 50°. Other transitions are also indicated. The phosphorescence oscillator (^3L_a) points perpendicular to both indole and isoalloxazine planes and is unfavourably oriented for energy transfer to the flavin. Details concerning the assignments are given in [17]

Although it cannot be stated with certainty, the results indicate that it is more probable that only one tryptophan participates in energy transfer. Taking into consideration the conclusion that the indole and flavin rings are fixed and oriented in one plane it can be calculated [18] from the polarization value of the flavin fluorescence excited at 295 nm that the angle between the tryptophan excitation vector and the flavin emission vector is approximately 50° (Fig. 6). A distance can be estimated [37]

by measuring the energy transfer efficiency from the relative intensities of holoenzyme and FAD-excitation spectra plus the normalized holoenzyme absorption spectrum at 295 nm. The efficiency was found to be 40—50% and is about twice as large when only one tryptophan transfers energy to the flavin. From Eqn (3) and the data in Table 2 with $\varkappa^2 = 2/3$, the distance between the donor and the acceptor was found to be 1.3—1.6 nm taking apoenzyme II and apoenzyme I, respectively, as basis for the calculation. It should be recognized that the assumptions are very crude and more information is necessary in order to obtain a good correlation between theory and experiment.

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A Pulse Fluorometry Study of Lipoamide Dehydrogenase

Evidence for Non-Equivalent FAD Centers

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The time dependence of the fluorescence of tryptophanyl and flavin residues in lipoamide dehydrogenase has been investigated with single-photon decay spectroscopy.

When the two FAD molecules in the enzyme were directly excited the decay could only be analyzed in a sum of two exponentials with equal amplitudes. This phenomenon was observed at 4 °C (τ_1 = 0.8 ns, τ_2 = 4.7 ns) and at 20 °C (τ_1 = 0.8 ns, τ_2 = 3.4 ns) irrespective of the emission and excitation wavelengths. This result reveals a difference in the nature of the two FAD centers.

By excitation at 290 nm the fluorescence decay curves of tryptophan and FAD were obtained. The decays are analyzed in terms of energy transfer from tryptophanyl to flavin residues. The results, which are in good agreement with those obtained previously with static fluorescence methods, show that one of the two tryptophanyl residues within the subunit transfers its excitation energy to the flavin located at a distance of 1.5 nm.

The luminescence properties of the apoprotein and holoprotein of lipoamide dehydrogenase from pig heart (NADH : lipoamide oxidoreductase) were investigated recently with static luminescence methods and with phase fluorometry [1]. One aspect of this study was the energy transfer process between tryptophan and flavin, from which an average distance in the order of 1.3 – 1.6 nm was estimated assuming one single donor-acceptor couple rigidly bound within the protein framework. However, only indications together with some crude calculations gave rise to the assumption that the energy transfer occurs between one pair of chromophores within the subunit. In the hope of getting additional support for previous conclusions, a pulse fluorometry study of the tryptophan and sensitized flavin fluorescence of the holoenzyme was performed.

A second problem, not directly connected with the first one, concerns the symmetry with regard to the two FAD sites of the dimeric holoenzyme. As judged from experiments of Massey *et al.* both active centers are considered to be identical [2, 3]. Fortunately, the oxidized protein-bound flavin itself shows in contrast to many other flavoproteins a considerable amount of fluorescence and can act as a natural probe for the microenvironment of the flavins provided that both FAD molecules are fluorescent. Therefore the fluorescence decay curves obtained by direct excitation of the flavin were closely analyzed in order to investigate whether a correlation exists between the equivalency of sites and the homogeneity of the fluorescence lifetime.

Abbreviations. FMN, flavin mononucleotide; FAD, flavin adenine dinucleotide; EDTA, ethylenediaminetetraacetic acid; τ, fluorescence lifetime.

Enzyme. Lipoamide dehydrogenase or NADH : lipoamide oxidoreductase (EC 1.6.4.3).

MATERIALS AND METHODS

Experimental Conditions

The preparation of lipoamide dehydrogenase from pig heart has been described previously [3]. Solutions of 10 µM enzyme in 0.03 M phosphate buffer of pH 7.2 containing 0.3 mM EDTA were used in this work. The experiments were performed at two temperatures, namely 4 °C and 20 °C.

Fluorescence Decay Measurements

Fluorescence decays were obtained with a single photoelectron counting apparatus described earlier [4]. A free-running flash lamp operating in nitrogen at high pressure was used. The excitation and emission wavelengths were selected by grating monochromators with bandwidths of 10 nm and 6 nm respectively. The response function $g(t)$ of the apparatus, which is used for the decay analysis, was obtained with a reference compound by a method described elsewhere [5]. The references used here were a solution of 1,1,4,4-tetraphenyl 1,3-butadiene in cyclohexane (time constant of 1.78 ns according to the unpublished results of J. C. Brochon) in the case of the flavin emission and a solution of *para*-terphenyl in cyclohexane in the case of the tryptophan emission (time constant 0.96 ns [5]). The compounds were obtained from Koch Light Laboratories Ltd.

Analysis of the data was performed with a computer program based on the method of modulating functions [7]. The program was designed to determine the three parameters relative to decays which are sums of two exponential terms according to the following expression:

$$I(t) = A[C_1\, e^{-t/\tau_1} + (1 - C_1)\, e^{-t/\tau_2}] \quad (1)$$

where A is a factor of normalization.

The fit of these parameters was checked by calculation the convolution of the decay, described by Eqn (1), with the apparatus response function, $g(t)$:

$$i^0(t) = \int_0^t g(T)\, I(t-T)\, dT. \quad (2)$$

This computed curve, $i^0(t)$, was then visually compared with the experimental curve, $i(t)$, and the weighted residue (R) was calculated according to a method described by Knight and Selinger [8]:

$$R = \frac{1}{n} \sum_{i=1}^{n} \frac{1}{i(t)} [i(t) - i^0(t)]^2. \quad (3)$$

In Eqn (3) it is assumed that the experimental decay curve, $i(t)$, is described by a distribution of counts into n channels. Theoretically it was shown [8] that the best computed curve is obtained when $R = 1$. In practice however good fits were obtained when R was around 2, while the range $R = 3-5$ resulted in reasonable fits.

In order to improve the solution a set of combinations of two time constants was tried, the values of which were chosen around the solution given by the modulation function method. The amplitude C_1 was calculated by using the expression which relates the quantities to the decay time constant and to the truncated moments [7]. The solution adopted was the one which had the smallest average weighted residue.

If we assume that the two flavins are located rigidly within the enzyme while both molecules can be considered as independent and non-interacting several special cases can be distinguished.

a) The two flavins have identical surroundings and can be considered as indistinguishable. Both FAD molecules should have the same fluorescence lifetime and Eqn (1) reduces to a single exponential term.

b) The two flavins have different environments, but the absorption spectra are (almost) equivalent. If one of the flavins is fluorescent and the other not one-exponential decay must be expected. If both FAD molecules are fluorescent but with different quantum yields the decay can be described by Eqn (1). If, in addition, the shape of the individual fluorescence spectra is also identical, there will be no variation of C_1 with the emission wavelength. In this special ideal case the amplitude C_1 is a direct measure for the amount of emitting species and should be equal to 0.5. If the individual flavin emissions are spectrally different, C_1 should be a function of the emission wavelength.

Fluorescence Decay in the Case of Energy Transfer

The energy transfer process from a donor, tryptophan, to an acceptor, FAD, has been shown to exist in the holoprotein [1,9]. In the simple case where there is only one kind of donor-acceptor couple the time dependence of the donor fluorescence after an infinitely short excitation (δ-pulse) can be described by [10,11]:

$$I_D(t) = I_D(0)\, e^{-t/\tau_D} \quad (4)$$

with

$$1/\tau_D = 1/\tau_{D0} + K_{DA} \quad (5)$$

where τ_{D0} is the lifetime of the donor fluorescence in the absence of the acceptor and K_{DA} is the transfer rate constant. The value of K_{DA} depends on the relative distance and orientation between donor and acceptor and can be represented as:

$$K_{DA} = 1/\tau_{D0}\, (R_0/r)^6 \quad (6)$$

where R_0 is the critical transfer distance and r is the distance between donor and acceptor [11,12].

The time dependence of the fluorescence of the acceptor after δ-pulse excitation can be shown (*cf.* [10]) to be proportional to:

$$I_A(t) \sim (1/\tau_A - 1/\tau_D)^{-1}\, (e^{-t/\tau_A} - e^{-t/\tau_D}) \quad (7)$$

where τ_A is the lifetime of the acceptor fluorescence obtained by direct excitation.

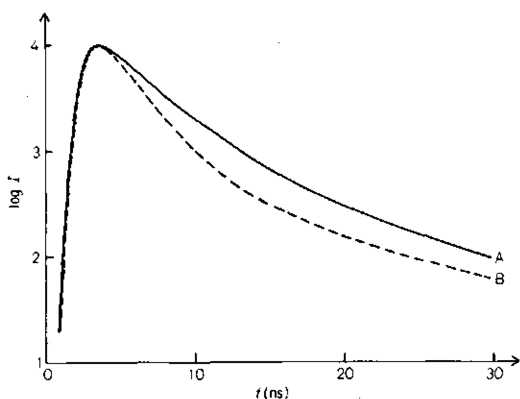

Fig. 1. *Fluorescence decay of (A) lipoamide dehydrogenase and (B) tetraphenyl-butadiene.* λ_{ex} = 370 nm, λ_{em} = 520 nm. Temperature = 20 °C

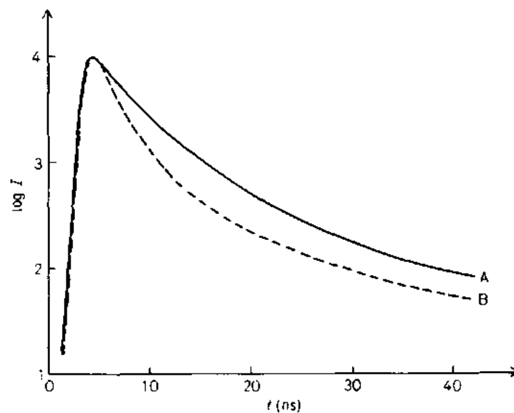

Fig. 2. *Fluorescence decay of (A) lipoamide dehydrogenase and (B) p-terphenyl.* λ_{ex} = 290 nm, λ_{em} 340 nm. Temperature = 20 °C

Table 1. *Parameters defining the fluorescence decay of the flavin in lipoamide dehydrogenase excited in the second absorption band of FAD*
Temperature 20 °C. R = average weighted residue. Details are given in the text.

λ_{ex}	λ_{em}	τ_1	τ_2	C_1	R
nm		ns			
330	520	0.8	3.6	0.51	4.2
350	520	1.0	3.6	0.53	5.1
370	520	0.8	3.2	0.46	2.2
370	500	0.8	3.4	0.50	4.3
370	520	0.8	3.2	0.46	2.2
370	540	0.8	3.4	0.48	4.7
370	560	0.8	3.4	0.49	4.3

Table 2. *Parameters defining the fluorescence decay of lipoamide dehydrogenase excited in the second absorption band of FAD*
Temperature 4 °C. R = average weighted residue. Details are given in the text

λ_{ex}	λ_{em}	τ_1	τ_2	C_1	R
nm		ns			
330	520	1.2	4.9	0.45	3.2
350	520	0.6	4.5	0.51	2.6
370	520	0.8	4.7	0.44	5.0
370	500	0.85	4.7	0.49	3.4
370	520	0.8	4.7	0.44	5.0
370	540	0.6	4.7	0.47	3.7
370	560	1.0	4.7	0.45	3.5

Since the enzyme contains four tryptophans and two FAD molecules, one could expect that the decays are linear combinations of Eqns (4) and (7).

RESULTS

Direct Excitation of the Flavin

The reference compound, 1,1,4,4-tetraphenyl 1,3-butadiene, does not absorb light in the wavelength range corresponding to the first absorption band of the flavin (> 400 nm). Therefore wavelengths within the second absorption band were selected for direct excitation of the flavin. The excitation and emission wavelengths were varied systematically and the fluorescence decay curves were analyzed as described above. A typical example is given in Fig. 1. In all cases the decay could not be described by a single exponential function, but good fits were obtained with a sum of two exponential terms. The time constants and the relative amplitude C_1 [*cf.* Eqn (1)] are collected in Tables 1 and 2 for 20 °C and 4 °C respectively. It should be noted that there is no systematic variation of the decay parameters with the changes of excitation and emission wavelengths.

At 20 °C the average parameters are: τ_1 = 0.8 ns; τ_2 = 3.4 ns; C_1 = 0.49; C_2 = 0.51 and at 4 °C: τ_1 = 0.8 ns; τ_2 = 4.7 ns; C_1 = 0.45; C_2 = 0.55.

Excitation at 290 nm

By excitation at 290 nm two emission bands can be observed in the fluorescence spectrum: the first one with its maximum located at about 330 nm is due to the tryptophans, which absorb most of the light

Table 3. *Parameters defining the fluorescence decays of lipoamide dehydrogenase excited at 290 nm*
R = average weighted residue. Details are given in the text

Temp.	λ_{ex}	λ_{em}	τ_1	τ_2	C_1	R
°C	nm		ns			
20	290	340	0.6	4.2	0.86	3.8
4	290	340	0.8	5.0	0.85	5.3
20	290	520	1.8	3.3	0.45	2.0

and the second one originates from the flavin (maximum at about 520 nm) mainly by energy transfer from tryptophanyl residues [1]. Fig. 2 shows the decay at 340 nm in comparison with the decay of the fluorescence of *p*-terphenyl. Also in this case is the best fit of the experimental curve a convolution of $g(t)$ with a sum of two exponential terms. The parameters are given in Table 3.

DISCUSSION

A very interesting outcome of this study is that the two FAD sites seem to be non-equivalent, as can be concluded from the fluorescence decay measurements of the flavin part of the enzyme. Generally, for a population of emitting species having different time constants, the fluorescence decay is a sum of exponential terms. The relative amplitudes are proportional to the molar fractions of these different species. There are some restrictions to that last statement. In particular no energy transfer should occur between the chromophores of different species. In the present case this means that energy transfers between the two FAD molecules bound to the same enzyme must be insignificant. This condition seems to be effectively realized since on one side the overlap integral between the corresponding absorption and emission spectra is small and on the other side the fluorescence polarization is high [1]. In principle, however, it can be expected that the flavin dinucleotide exists at least in two conformations (open and closed), which are in equilibrium. As was shown earlier the flavin fluorescence of free FAD in aqueous solution has one single time constant which value is strongly temperature dependent [13, 14]. Only the open conformation is fluorescent. If, in the case of the enzyme, the double exponential decay of the flavin fluorescence was the result of two different FAD conformations in equilibrium, the ratio of the amplitudes of the two contributions in the decay must be expected to vary with the temperature. However, the results show that this does not occur within the limit of experimental accuracy. In addition the ratio of these amplitudes is close to one. It thus seems reasonable to conclude that the two kinds of FAD are related with the two independent sites of the enzyme. A fast dynamic motion of the isoalloxazine part is improbable, because the high value of the degree of polarization of the fluorescence indicates a rigidly bound flavin [1].

There is no systematic variation in the results with varying excitation and emission wavelengths, which implies that the shape of the emission and (second) absorption bands of the two species are identical within experimental error. If the quantum yields of the two FAD molecules are supposed to be proportional to their lifetimes the ratio of the two quantum yields is then equal to 4 at 20 °C and to 6 at 4 °C. Data of De Kok *et al.* [15] show that the quantum yield of the flavin fluorescence of the enzyme is about half of that of FMN at 4 °C. The corresponding fluorescence lifetimes are 4.2 ns for the enzyme and 4.9 ns for FMN. At 25 °C the relative yield is about one third, while the lifetime is 2.3 ns (for FMN 4.7 ns). These lifetime values, obtained by phase fluorometry, can be compared with the average lifetimes ($<\tau>$), obtained with the single-photon counting technique as described earlier [14]. At 4 °C $<\tau>$ is found to be 4.1 ns and at 20 °C 2.8 ns. It should be realized that in this case of heterogeneous emission the most predominant contribution in the results obtained by phase fluorometry is given by the component with the longest lifetime and largest quantum yield, whereas the results obtained by pulse fluorometry are in better agreement with reality. These experiments indicate the presence of two kinds of FAD, which are both fluorescent having different lifetimes and therefore different quantum yields. It has been shown that the values of the quantum yield and lifetime of FMN are, in contrast to those of FAD, hardly dependent on the temperature [13, 14]. If the FAD component in lipoamide dehydrogenase with the longest lifetime (τ_2) at 4 °C is connected with a flavin in a FMN-like or open conformation the relative large change of τ_2 with temperature must be attributed to a dynamic quenching of the flavin fluorescence. This could be due to the adenine moiety itself or to a protein residue situated in the vicinity of the isoalloxazine ring.

From earlier experiments it was concluded that the two subunits and flavin sites seem to be equivalent [2, 3]. The present results, however, show that the two FAD centers have different microenvironments, which implies that the overall symmetry of the dimeric enzyme is not two-fold. It is not completely clear yet whether such asymmetry could explain some aspects of the enzymatic reaction. By kinetic and spectral methods cooperative interaction of the sub-

strates with the enzyme has been shown to be present, indicating that the two centers are not independent [16–19]. It should be interesting to investigate whether the substrates show preference for one of the two binding sites.

The experimental results obtained by excitation at 290 nm are not easy to explain. *A priori*, the chromophoric system which leads to these emissions is not a simple one. The enzyme contains four tryptophanyl residues. Under favourable conditions, each of these residues could have its own fluorescence lifetime and could transfer its energy to the two flavin compounds in the protein. The fluorescence response function at 340 nm can be expected to be composed of a sum of four exponential terms. This number can be reduced by the presence of some symmetry in the dimeric protein molecule. The two decay times, which are observed, can probably be considered as average values. The main contribution in the experimental decay is characterized by a short time constant (0.6 ns) at 20 °C. This value is smaller than the average value of 1.6 ns, which was earlier obtained by phase fluorometry [1], and is indicative for fast energy transfer. Taking the lifetime of the tryptophan fluorescence of apoenzyme I as 3.8 ns [1], an average transfer rate can be calculated as 1.4 ns^{-1} by applying Eqn (5). This value is a factor three larger than that obtained previously, which is due to the very short time constant measured by the single-photon counting technique. After applying Eqn (6) with R_0 as 2.0 nm [1], an average distance r can be calculated as 1.5 nm, which is in close correspondence with the previous value of 1.6 nm [1]. From Table 3 it can be concluded that 15% of the tryptophanyl residues are characterized by a longer time constant of 4.2 ns, belonging to residues showing an insignificant transfer efficiency (see below). According to Eqn (7) the decay of the flavin fluorescence excited by energy transfer, is the sum of the exponential terms. The time constants are the decay times of the transferring tryptophans and of the flavins (as measured by direct flavin excitation). The decay should be a complicated one involving negative amplitudes. Consequently, the following considerations based on the resolution into two exponential terms as is presented in Table 3, are rather qualitative in nature.

Firstly, it should be noted that no time constant of about 4.2 ns appears in the flavin fluorescence decay. This justifies the conclusion that the corresponding tryptophans have practically no transfer efficiency. The conclusion that not all tryptophanyl residues in the holoenzyme participate in energy transfer was also obtained earlier in an indirect way [1]. Secondly the long time constant found in this transfer analysis is practically identical to the one found upon direct excitation of the flavin. The site characterized by this constant must be an acceptor of energy transfer. Finally the short constant of indirect flavin emission decay is longer than the short time constant obtained upon direct flavin excitation. This must be due to the delay caused by the time needed for transfer of the energy from tryptophan to FAD. This is not an unreasonable explanation bearing in mind that the time resolution of the apparatus as well as the time scale of energy transfer are both in the subnanosecond region.

CONCLUSION

Perhaps the most important result of this work is that the two FAD molecules bound to the enzyme are characterized by two different fluorescence lifetimes. This implies different conformations of the two flavin sites, which might have consequences for the reaction mechanism. It should be interesting to see if this phenomenon also exists in other flavoproteins, because many of these enzymes have a dimeric structure.

Recently, evidence has been given that such dissymmetry also occurs in other protein systems [20]. From electronic absorption spectra it was concluded that the two iron (III) sites in spinach ferredoxin are not equivalent.

The energy transfer experiments seem to justify the previous conclusion that not all the tryptophans are involved in energy transfer to the flavin. The distance between a tryptophan flavin couple, calculated from the transfer rate, is in good agreement with the value obtained earlier from the transfer efficiency [1].

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APPENDIX 1

Derivation of the two-exponential decay in the scheme (fig. 1) of paper II

Following the kinetic scheme in figure 1 of paper II the rate equations important for the fluorescence after δ-pulse excitation can be written in matrix form as two simultaneous differential equations with constant coefficients.

$$\begin{pmatrix} \frac{d}{dt} + k_1 + k_+^* & -k_-^* \\ -k_+^* & \frac{d}{dt} + k_2 + k_-^* \end{pmatrix} \begin{pmatrix} |A_1^*| \\ |A_2^*| \end{pmatrix} = 0 \qquad A_{1-1}$$

with general solutions:

$$|A_1^*|\ (t) = C_1 e^{-t/\tau_1'} + C_2 e^{-t/\tau_2'}$$
$$|A_2^*|\ (t) = C_3 e^{-t/\tau_1'} + C_4 e^{-t/\tau_2'} \qquad A_{1-2}$$

τ_1' and τ_2' are interrelated by:

$$1/\tau_1' \cdot 1/\tau_2' = k_1 k_2 + k_2 k_+^* + k_1 k_-^*$$
$$1/\tau_1' + 1/\tau_2' = k_1 + k_2 + k_+^* + k_-^* \qquad A_{1-3}$$

The fluorescence intensity is related to:

$$I_{A_1}(t) = k_{f_{A_1}} |A_1^*|(t) = k_{f_{A_1}} (C_1 e^{-t/\tau_1'} + C_2 e^{-t/\tau_2'})$$

$$I_{A_2}(t) = k_{f_{A_2}} |A_2^*|(t) = k_{f_{A_2}} (C_3 e^{-t/\tau_1'} + C_4 e^{-t/\tau_2'}) \qquad A_{1-4}$$

$k_{f_{A_1}}$ and $k_{f_{A_2}}$ are the radiative rate constants for species A_1 and A_2 respectively.
The equations A_{1-4} are of the same form as those derived for excimer and exciplex formation, protonation in the excited state or quenching by other molecules |1-4|.
C_1, C_2, C_3 and C_4 are dependent and can be derived from the initial boundary conditions:

$$\text{on } t = 0 \text{ is } |A_1^*| = |A_{10}^*| = \varepsilon_A I_o |A_1|$$
$$\text{and } |A_2^*| = |A_{20}^*| = \varepsilon_B I_o |A_2| \qquad A_{1-5}$$

ε_{A_1} and ε_{A_2} are the molar decadic extinction coefficients of both forms at the excitation wavelength in 1 $mole^{-1}cm^{-1}$ and I_o is the intensity of the exciting light in Einsteins $1^{-1}sec^{-1}$.

$|A_1|$ and $|A_2|$ can be expressed in the total flavin concentration in the ground state. $|F| = |A_1| + |A_2|$, and in the equilibrium constant in the ground state $K = |A_2|/|A_1|$. It is assumed further that $\varepsilon_{A_1} \approx \varepsilon_{A_2} = \varepsilon$, which is allowed if the excitation wavelength is at an isosbestic point. Furthermore since the absorption spectra in the visible and near U.V. region of FAD and FMN are only slightly different, indicating weak interaction between adenine and isoalloxazine it is assumed that $k_{f_{A_1}} = k_{f_{A_2}} = k_f$ |5|. With these assumptions C_1, C_2, C_3 and C_4 can be solved via A_{1-1} and A_{1-2}.

$$C_1 = P\,(1/\tau_2' - k_1 - k_+^* + k_-^* K)$$

$$C_2 = P\,(k_1 + k_+^* - k_-^* K - 1/\tau_1')$$

$$C_3 = P\,(K/\tau_2' - k_2K - k_-^* K + k_+^*) \qquad A_{1-6}$$

$$C_4 = P\,(k_2K + k_-^* K - k_+^* - K/\tau_1')$$

$$\text{with } P = \frac{\varepsilon\, I_o|F|}{1/\tau_2' - 1/\tau_1'}$$

The time dependence of the total fluorescence is $I(t) = I_{A_1}(t) + I_{A_2}(t)$, which can be shown to be

$$I(t) = \frac{k_f\, \varepsilon\, I_o|F|}{1/\tau_1' - 1/\tau_2'} \{(1/\tau_2' - M)\, e^{-t/\tau_1'} + (M - 1/\tau_1')\, e^{-t/\tau_2'}\} \qquad A_{1-7}$$

$$\text{with } M = \frac{k_1 + k_2K}{K + 1}$$

Thus the decay of the fluorescence is the sum of two exponentials with different amplitudes.

From an analysis of the fluorescence decay it is possible to determine τ_1', τ_2' and the amplitude ratio:

$$C = \frac{1/\tau_2' - M}{M - 1/\tau_1'} \qquad A_{1-8}$$

One has to know one of the rate constants, k_1 or k_2, to evaluate the other from M, provided that the association constant in the ground state, K, is known by other means (e.g. via absorption spectrophotometry or nuclear magnetic resonance). From A_{1-3} the other rate constants can then be determined.

Wavelength dependence

Suppose that the individual emission spectra of A_1 and A_2 are different and overlapping. The wavelength dependence of $I_{A_1}(t)$, $I_{A_2}(t)$ and $I(t)$ can then be written as:

$$I_{A_1}(\lambda, t) = k_f C_{A_1}(\lambda)\ I_{A_1}(t)$$

$$I_{A_2}(\lambda, t) = k_f C_{A_2}(\lambda)\ I_{A_2}(t) \qquad A_{1-9}$$

$$\begin{aligned} I(\lambda, t) &= k_f C_{A_1}(\lambda)\ I_{A_1}(t) + k_f C_{A_2}(\lambda)\ I_{A_2}(t) \\ &= k_f\{(C_{A_1}C_1 + C_{A_2}C_3)\ e^{-t/\tau_1'} + (C_{A_1}C_2 + C_{A_2}C_4)\ e^{-t/\tau_2'}\} \end{aligned}$$

with the normalization condition $\int_0^\infty C_{A_1}(\lambda)\ d\lambda = \int_0^\infty C_{A_2}(\lambda)\ d\lambda = 1.$

The terms $(C_{A_1}C_1 + C_{A_2}C_3)$ and $(C_{A_1}C_2 + C_{A_2}C_4)$ are the same as the amplitudes $A_1'(\lambda)$ and $A_2'(\lambda)$ of paper II.
If the equations in A_{1-9} are integrated over time the individual time independent emissions at wavelength λ are obtained. These emissions can be measured in the conventional way with continuous excitation.
Thus

$$I_{A_1}(\lambda) = k_f C_{A_1}(C_1\tau_1' + C_2\tau_2')$$

$$I_{A_2}(\lambda) = k_f C_{A_2}(C_3\tau_1' + C_4\tau_2') \qquad A_{1-11}$$

$$I(\lambda) = I_{A_1}(\lambda) + I_{A_2}(\lambda) = k_f\{C_{A_1}(C_1\tau_1' + C_2\tau_2') + C_{A_2}(C_3\tau_1' + C_4\tau_2')\}$$

It can be shown that:

$$I_{A_1}(\lambda) = \frac{(CC_4 - C_3)(C_1\tau_1' + C_2\tau_2')}{(C\tau_1' + \tau_2')(C_1C_4 - C_2C_3)}\ I(\lambda) \qquad A_{1-12}$$

and for species A_2:

$$I_{A_2}(\lambda) = I(\lambda) - I_{A_1}(\lambda)$$

$I(\lambda)$ can be measured for the emission spectrum; all the constants in A_{1-12} can be calculated from the rate constants, which are determined from the fluorescence decay analysis.
It should be noted that the common factor P of A_{1-6} is eliminated in A_{1-12}. Integrating of the equations in A_{1-11} over wavelength should give the area under the emission spectra which is proportional to the quantum yields:

$$\int_0^\infty I_{A_1}(\lambda)\, d\lambda \sim C_1\tau_1' + C_2\tau_2' = S_{A_1}$$

$$\int_0^\infty I_{A_2}(\lambda)\, d\lambda \sim C_3\tau_1' + C_4\tau_2' = S_{A_2} \qquad A_{1-14}$$

$$\int_0^\infty I(\lambda)\, d\lambda \sim S = S_{A_1} + S_{A_2}$$

Special cases

The rather complicated picture can sometimes be simplified.

a. The second form A_2 is nonfluorescent (dark), so that $1/\tau_2' \to \infty$. The fluorescence is due to the open form A_1 and its decay is expected to be exponential with a rate constant of $1/\tau_1' \approx k_1 + k_+^*$. This is probably the situation in FAD.

b. $k_+^*, k_-^* << k_1, k_2$.
This means slow equilibrium in the excited state, the equilibrium in the ground state is not perturbed.
Neglecting all terms in k_+^* and k_-^* with respect to k_1 and k_2 (cf. A_{1-3}) it can be shown that $1/\tau_1' = k_1$ and $1/\tau_2' = k_2$ so that

$$I(\lambda, t) \sim e^{-k_1 t} + K\, e^{-k_2 t}$$

and

$$I_{A_1}(\lambda) = \frac{k_2}{k_2 + Kk_1} I(\lambda)$$

and $C = 1/K$

c. The same situation as in b, except that A_2 is dark, so that

$$I(\lambda, t) = I_{A_1}(\lambda, t) \sim e^{-k_1 t} \qquad \text{(exponential decay)}$$

d. $k_+^*, k_-^* >> k_1, k_2$.
This is fast equilibrium in the excited state.
Neglecting the corresponding terms in A_{1-3} it can be shown that

$$1/\tau_1' = 1/\tau_2' = 1/\tau \approx \frac{k_2 + k_1 K_e}{K_e + 1}$$

with $K_e = k_-^*/k_+^*$.

The emissions of A_1 and A_2 decay exponentially with the same time constant τ.

e. As in d, but A_2 is dark. Only the open form is fluorescent with a single lifetime.

Flavin fluorescence decay of a dimeric flavoprotein

The fast dynamic equilibrium in free FAD is not probable in FAD, which is bound tightly to a protein. One conformation is predominant excluding an equilibrium of the type in fig. 1 of paper II. In dimeric flavoproteins two FAD molecules are present, which do not need to be identical. For two non-interacting, inequivalent and fluorescent FAD molecules the flavin fluorescence decay is heterogeneous and can be described generally by a sum of two exponential terms:

$$I(\lambda, t) \sim C_1(\lambda, t)\, e^{-k_1 t} + C_2(\lambda, t)\, e^{-k_2 t} \qquad A_{1-15}$$

k_1 (= $1/\tau_1$) and k_2 (= $1/\tau_2$) are the rate constants for fluorescence of FAD species 1 and 2 respectively; C_1 and C_2 are their relative amplitudes. Several cases can be distinguished.

a) The individual absorption and emission spectra are identical, but the fluorescence quantum yields are different. A light pulse excites equal populations of both species, so that directly after the pulse equal concentrations of components 1 and 2 are present; the emissions of each component decay independently with time constants τ_1 and τ_2. the total fluorescence decay obeys equation A_{1-15}. C_1 and C_2 show no dependence on emission and excitation wavelengths. The experiments indicate that this situation is approximated in lipoamide dehydrogenase.

b) The absorption spectra, emission spectra and quantum yields are different. The total decay is described by equation A_{1-15}, but C_1 and C_2 are functions of excitation and emission wavelengths.

Variations between these two extreme situations are, of course, possible. Single exponential decay is expected if one of the two species is nonfluorescent (dark) or if both flavins are spectrally indistinguishable and equivalent.

Flavin fluorescence decay of a dimeric flavoprotein as studied by the phase and modulation technique /6/.

A population of molecules is excited by sinusoidally modulated light of a fixed angular frequency ω described by:

$$E(t) = A + B \cos\omega t \qquad A_{1-16}$$

The degree of modulation is B/A.

The fluorescence response on this periodic function is given by (for an exponential decay):

$$I(t) = A + B \cos\delta \cos(\omega t - \delta) \qquad A_{1-17}$$

The degree of modulation is now $B \cos\delta/A$.
The phase angle δ is related to the lifetime τ via

$$\tan \delta = \omega\tau \qquad A_{1-18}$$

The modulation depth (M) can be defined by

$$M = \frac{B \cos\delta}{A} \bigg/ \frac{B}{A} = \cos\delta = (1 + \omega^2\tau^2)^{-\frac{1}{2}} \qquad A_{1-19}$$

It can be derived from A_{1-18} and A_{1-19} that the fluorescence lifetime τ may be determined from independent measurements of phase angle δ (τ_p) and of modulation depth M (τ_m). For a homogeneous decay both measurements should give the same lifetime; for a heterogeneous emission τ_p and τ_m are different with $\tau_m > \tau_p$. This inequality can be derived from the average values of τ_p ($<\tau_p>$) and τ_m ($<\tau_m>$) of a double exponential decay:

$$<\tau_p> = \frac{1}{\omega} \times \frac{C_1/C_2 \sin\delta_1 \cos\delta_1 + \sin\delta_2 \cos\delta_2}{C_1/C_2 \cos^2\delta_1 + \cos^2\delta_2} \qquad A_{1-20}$$

$$<\tau_m> = \frac{1}{\omega} \left\{\frac{1}{(C_1 \cos^2\delta_1 + C_2 \cos^2\delta_2)^2 + (C_1 \cos\delta_1 \sin\delta_1 + C_2 \cos\delta_2 \sin\delta_2)^2} - 1\right\}^{\frac{1}{2}} \qquad A_{1-21}$$

δ_1 and δ_2 are the phase angles of components 1 and 2 respectively; C_1 and C_2 are their fractional intensities.
It should be realized that the intensity ratio C_1/C_2 is different from the amplitude ratio C_1/C_2 in equation A_{1-15}. This has to do with the different modes of excitation used in the two type of experiments.

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APPENDIX 2

Fluorescence decay in the case of dipole-dipole energy transfer

Förster has derived the phenomenological expression for the rate constant of nonradiative energy transfer from donor to acceptor molecules in the weak coupling limit |1|:

$$K_{DA} = 1/\tau_{D_o} \, (R_o/r)^6 \qquad A_{2-1}$$

(cf. equations 4 (paper III) and 6 (paper IV)).
Försters theory of resonance energy transfer was confirmed in a series of very elegant experiments |2|.
From equation A_{2-1} it is possible to estimate the donor-acceptor distance in a protein system if the other parameters are known. These can be determined from spectral data and from fluorescence decay analysis.
Suppose a binary system containing donor and acceptor molecules, the lowest excited singlet state of the donor being higher in energy than that of the acceptor. As pointed out by Birks two ultimate cases can be distinguished |3|:

a) In solutions of low viscosity complete mixing of donor and acceptor molecules occurs during the transfer process; the rate constant of energy transfer is time independent. The system is said to obey "Stern-Volmer" kinetics.
b) In solutions of high viscosity donor and acceptor molecules remain stationary and randomly oriented during the transfer process; the transfer rate becomes time dependent. The system is said to obey "Förster" kinetics.

These ultimate cases were taken as starting points for the development of theories concerning the role of diffusion in dipole-dipole energy transfer (for references see ref. 3, p. 577).

<u>Ad a</u>

The rate equations describing the time dependence of the donor and acceptor concentrations in the excited states after δ-pulse excitation are in matrix form:

$$\begin{pmatrix} \frac{d}{dt} + K_{D_o} + K_{DA} & 0 \\ - K_{DA} & \frac{d}{dt} + K_A \end{pmatrix} \begin{pmatrix} |D^*| \\ |A^*| \end{pmatrix} = 0 \qquad A_{2-2}$$

where K_{D_o} $(= 1/\tau_{D_o})$ is the rate constant of the donor fluorescence in the absence of transfer, K_{DA} $(= K'_{DA}|A|)$ is the rate constant for energy transfer and K_A is the rate

constant of the acceptor fluorescence, when the acceptor is excited directly; it is convenient to substitute $K_D = 1/\tau_D = K_{D_o} + K_{DA}$.
After following a similar treatment as in appendix 1 for the derivation of the two-exponential decay the fluorescence response of the donor to a δ-pulse can be represented by:

$$I_D(t) = K_{F_D} |D_o^{*}| \, e^{-t/\tau_D} \qquad A_{2-3}$$

and of the acceptor, which has been excited via energy transfer:

$$\underset{(D \rightarrow A)}{I_A(t)} = \frac{K_{F_A} K_{DA} |D_o^{*}|}{1/\tau_D - 1/\tau_A} \left(e^{-t/\tau_A} - e^{-t/\tau_D}\right) \qquad A_{2-4}$$

or $\underset{(D \rightarrow A)}{I_A(t)} \sim I_A(t) - I_D(t)$

K_{F_D} and K_{F_A} are the radiative rate constants for donor and acceptor respectively, $|D_o^{*}|$ is the donor concentration in the excited state on t = 0, $I_A(t)$ represents the fluorescence decay of the acceptor, which has been excited directly.
Equations A_{2-3} and A_{2-4} are similar to equations 4 and 7 (paper IV).
By measuring the fluorescence lifetime of the donor in the presence (τ_D) and in the absence (τ_{D_o}) of acceptor molecules K_{DA} can be determined.

Ad b

Förster has derived an expression for the fluorescence decay of the donor in the presence of energy transfer; the rotatory Brownian motion of the donor molecules is considered to be fast, but the translational Brownian diffusion of donor and acceptor molecules is insignificant |4|:

$$I_D(t) = K_{F_D} |D_o^{*}| \, e^{\{-t/\tau_{D_o} - 2\gamma \, (t/\tau_{D_o})^{\frac{1}{2}}\}} \qquad A_{2-5}$$

where $\gamma = \dfrac{2\, \pi^{3/2} \, N \, R_o^3 \, |A|}{3000}$

N = Avogadro's number, R_o = critical transfer distance and |A| = the acceptor concentration; for a fixed acceptor concentration γ depends only on R_o.

By measuring the fluorescence decay of the donor in the absence of acceptor molecules τ_{D_o} can be determined; in the presence of a fixed acceptor concentration the decay can be analyzed to yield γ (and R_o).
Steinberg has derived a theoretical expression for the case that also the rotatory Brownian motion is frozen; the time dependence has a similar form as equation A_{2-5}, but the extent of transfer is less |5|. Bennett was the first who verified

experimentally equation A_{2-5} for a randomly oriented donor-acceptor system in rigid solution using a pulsed excitation source |6|. The fluorescence response of function of the acceptor excited via energy transfer has a far more complicated form. In principle it can be solved from the following differential equations:

$$\begin{pmatrix} \frac{d}{dt} + K_{D_o} + \gamma(K_{D_o} t)^{\frac{1}{2}} & 0 \\ -\gamma(K_{D_o} t)^{\frac{1}{2}} & \frac{d}{dt} + K_A \end{pmatrix} \begin{pmatrix} |D^*| \\ |A^*| \end{pmatrix} = 0 \qquad A_{2-6}$$

Details of the solving procedure can be found elsewhere 7 ; the result is:

$$\underset{D \to A}{I_A(t)} = K_{F_A} |D_o^*| \pi^{\frac{1}{2}} m e^{m^2} \{\mathrm{erf}(a^{\frac{1}{2}} t^{\frac{1}{2}} + m) - \mathrm{erf}\, m\} e^{-t/\tau_A} \qquad A_{2-7}$$

where $m = \left(\frac{K_{D_o}}{K_{D_o} - K_A}\right)^{\frac{1}{2}} \gamma$, $a = K_{D_o} - K_A$ and

$$\mathrm{erf}\, m = 2\pi^{-\frac{1}{2}} \int_0^m e^{-x^2} dx \text{ , the error integral.}$$

For large m (i.e. large γ or large R_o) the error integral can be expanded into a series:

$$\mathrm{erf}\, m = 1 - \frac{e^{-m^2}}{\pi^{\frac{1}{2}} m} \left(1 - \frac{1}{2m^2} \ldots.\right) \qquad A_{2-8}$$

Substituting the first two terms of A_{2-8} into A_{2-7} gives:

$$\underset{D \to A}{I_A(t)} \simeq K_{F_A} |D_o^*| \left(e^{-t/\tau_A} - e^{\{-t/\tau_{D_o} - 2\gamma(t/\tau_{D_o})^{\frac{1}{2}}\}}\right) \qquad A_{2-9}$$

or $\underset{D \to A}{I_A(t)} \sim I_A(t) - I_D(t)$, which has the same form as equation A_{2-4}.

Remarks

Lipoamide dehydrogenase is a complicated system in which to study energy transfer because the four donor and two acceptor molecules seem to occupy nonequivalent positions into the protein. Each donor-acceptor couple might have a different R_o-value and distance. In principle the contribution in the energy transfer of each couple should be included; the overall transfer rate is in fact a superposition of individual transfer rates. In the case of a random ensemble of donor and acceptor molecules there is a continuous distribution of rate constants, which means that the fluorescence decay of the donor is non-exponential. Fluorescence decay curves of donor and indirectly excited acceptor should be analyzed then with equations A_{2-5} and A_{2-7}. However, doubt exists, whether a protein

system containing a limited number of chromophores can be considered as a random ensemble of donor and acceptor molecules. As is discussed and demonstrated in papers III and IV, not all the tryptophans are involved in energy transfer to the flavin. A homogeneous population of these nontransferring molecules gives rise to an exponential decay with a characteristic time constant. This time constant should be present in the total decay of the donor fluorescence, but absent in the decay of the acceptor excited by energy transfer (cf. table 3 of article IV). In this case the number of donor molecules, which contributes effectively to energy transfer becomes less so that in lipoamide dehydrogenase probably less than the four tryptophans are transferring energy to the two flavins. This implies that in the protein each transferring donor molecule is in the same geometrical configuration with respect to the acceptor molecule. This gives rise to one single rate constant for energy transfer and the fluorescence decay curve of the (transferring) donor is expected to be exponential. It is then completely allowed to make use of equations A_{2-3} and A_{2-4} as is done in paper IV. Because the subunits do not seem to be identical with respect to the flavins, linear combinations of these equations might be used.

Since the donor fluorescence decay in the holoenzyme is built up from at least two exponentials (cf. table 3 of article IV), it can be explained using equation A_{1-20}, that the average lifetime measured with the phase fluorometer (τ_p) is longer than τ_1 and shorter than τ_2 of the donor fluorescence decay.

Georghiou has studied the effect of resonance energy transfer on the donor fluorescence decay with the phase and modulation technique |8|. The expressions are rather intricate and fall outside the scope of this appendix.

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DISCUSSION

At first sight the results obtained in this work do not seem to correlate with each other, but the contrary is true. It must be kept in mind that when the excited state of protein-bound flavin is investigated one needs exact knowledge about the excited state properties of the isolated flavin molecules in order to examine the influence of the protein on these properties. However one must be aware of complications, arising from the fact that free flavins in solution undergo some diffusion controlled processes, which are absent in protein bound flavins. The molecular motion of tightly bound flavins is mostly dominated by the much slower motion of the much larger protein molecules. These dynamic processes are at least partly responsible for the phenomena observed in papers I and II. The most significant features of papers I and II shall be discussed subsequently.
In paper I it can be noticed that the transient absorption difference spectra of species II and III decayed about 1000 times faster than the corresponding spectra of the flavin in plastic "solution". The reason for this rapid decay in fluid solution is that the flavin in its higher excited triplet state is now vulnerable for dynamic quenching processes, which diminishes the lifetime of the triplet state. As was not mentioned in paper I attempts were made to analyze the decay curves of the transient absorptions at different wavenumbers. Different concentrations of flavin (in the range 80 - 300 μM) and different laser energies (in the range 0.1 - 2 mJ) were used and the observation time was extended to 10 μsec after the laser pulse. In practical all cases no clear cut first or second order decay could be observed illustrating that the decay kinetics followed a complex mechanism. As also pointed out by Vaish and Tollin the decay of the flavin triplet is not only determined by the presence of quenchers (e.g. O_2) but also by intermolecular interactions with flavin in the ground as well as in the triplet state |1|. At low laser energy quenching by ground state flavin should be predominantly leading to a pseudo first order quenching process provided that the concentration of flavin triplet is sufficiently low with respect to its concentration in the ground state. This tendency is indeed observed in the more concentrated samples using a low laser energy. From the slope of a ln ΔA-time plot the rate constant for triplet-ground state quenching is approximately 2.0×10^8 $M^{-1}sec^{-1}$ (Vaish and Tollin found 3.7×10^8 $M^{-1}sec^{-1}$). This large value suggests very efficient quenching. The use of higher laser energies increases the population of flavins in the triplet state, so that triplet-triplet annihilation processes are favoured reducing the lifetime of the flavin in the triplet state |2|. Our data point in the same direction because at higher laser energies the decay becomes much faster. These "self quenching" processes are probably responsible for the fact that the transient absorptions observed in the laser experiments decayed more rapidly than the ones obtained from flash photolysis |1, 3, 4|. Because of the different experimental set up the concentrations of flavin used in the laser experiments were at least 4 - 10 times higher than the flavin concentrations

in photoflash experiments. In contrast with the flavin in solution the flavin in plastic showed transient absorption difference spectra with first order decay kinetics.
The presence of dimers is an attractive assumption for the explanation of the nature of transient III, but it should be realized that only circumstantial, not direct evidence was presented. It is specially difficult to imagine that at low flavin concentrations (20 μM) and in apolar solvents dimerisation can occur. However from the literature it is known that flavins are weakly interacting |5, 6|. Song et al. found dimer phosphorescence at low concentrations of flavin in ethanol or isopentane glasses |6|. The dimer phosphorescence in isopentane was more predominant than the comparable monomer emission even at concentrations of 30 μM of flavin |6|. If transient species III can be assigned to belong to a "photodimer", its molar extinction coefficient at 25,600 cm^{-1} should be higher than that of transient species II (flavin triplet) at the same wavenumber. It was excluded, that the excited singlet state of the flavin was involved because the lifetime and the quantum yield of the fluorescence were unaffected in the pH range 3-9, whereas transient species III showed a marked pH-dependence.
From flash photolysis data it is assumed that the absorption changes in the spectral region from 18,000 - 21,000 cm^{-1} can be ascribed partly to the flavin radical |1, 7|. This radical species could be generated in a bimolecular diffusion controlled reaction of flavins in the triplet and/or in the ground state; it is always present after a flash of 10 μsec duration |1, 7|. This reaction might occur in a photoflash experiment because of the long illumination time (10 μsec) as compared with the much shorter laser pulse (3 nsec). It is quite illustrating that the samples in flash photolysis experiments have to be refreshed very frequently because of permanent photobleaching. In laser flash experiments all the transient species were present directly after the pulse and no radical species with an absorption between 18,000 and 21,000 cm^{-1} was growing in. Moreover this spectral region was not affected by a change in pH over the range 2 - 9 whereas the flavin radical has a distinct pK value of 8.3, which should be noticed by its characteristic absorptions |8|. However, when an electron donating compound like EDTA (50 mM) was present in a neutral flavin solution, laser flash photolysis showed a very rapid triplet decay and a concomitant growing in (completed after 400 nsec) of flavin radical at 16,000 and 19,000 cm^{-1} (unpublished observations). It is believed that this example might illustrate that solid state lasers delivering a high amount of energy in a short pulse provide a powerful tool for the study of photoreactions.
The short study described in paper II is essential for a better understanding of the fluorescence properties of flavoproteins containing FAD. The most important outcome is that in aqueous solutions only one form of the internal complex, in this case the open conformation, gives rise to fluorescence. FAD can exist in more conformations which are in rapid equilibrium. It would be interesting to see if in apolar solvents, in which the dispersion forces between adenine and isoalloxazine rings are weaker, the fluorescence of more than one conformation can be detected.
Paper III contains the necessary information in which environments the tryptophan and flavin moieties are located in lipoamide dehydrogenase. It was a pity that addition of

increasing amounts of I^- or Br^- did not result in a proportional decrease of the flavin or tryptophan fluorescences. This collisional quenching method should elucidate the location of the chromophores (buried in or at the exterior of the protein), but these charged quenchers are not appropriate for this. The observation that negatively charged substrates (lipoic acid) have lower turnover numbers than uncharged substrates (lipoamide) might indicate that charged molecules are hindered to approach the active site |9|. The smaller Br^- ion does not give any flavin fluorescence quenching in contrast with the larger, more polarizable I^- ion. However, in the case of quenching with I^- the Stern-Volmer plot exhibits a "saturation pattern", which indicates that other quenching mechanisms are involved. The application of uncharged gaseous quenchers like oxygen (paramagnetic) or krypton (heavy atom) used under high pressure seems to be a powerful tool to obtain information concerning the location of chromophores in proteins |10, 11|. Using this technique, the environment of the two different FAD's in lipoamide dehydrogenase might be elucidated.
The results and the interpretation of the energy transfer experiments described in papers III and IV respectively must be considered as qualitative and approximate because the protein system contains 6 potential chromophores (the 14 tyrosins were not even taken in consideration). The apoenzymes must be used as reference not only for the calculation of R_0 but also to estimate the amount of energy transfer that is occurring. Although the emission spectra did not seem to differ very much, the protein conformations of both species (apo- and holoenzyme) were different. Furthermore, different apoenzymes can be prepared with different emission properties. These last two factors made it impossible to analyze the results very accurately.
One comment should be added to the use of equations 7 and 8 of paper III and equations 4 and 7 of paper IV. These equations are used for the case that the system obeys so-called Stern-Volmer kinetics |12|. This means that the donor and acceptor are diffusing so rapidly that i) the orientation factor κ^2 is averaged to 2/3 and ii) the rate of "statistical mixing" of donor and acceptor molecules exceeds the rate of energy transfer. K_{DA}, its rate constant, becomes then independent of time. This, of course, is not true for the system under study because the chromophores are fixed rigidly within the protein. Therefore the above mentioned expressions should be considered as a first approximation. For systems where the molecular motion of donor and acceptor molecules is frozen, it can be shown that the transfer rate constant, K_{DA}, becomes time dependent |13|. Förster has shown in this case that the donor fluorescence decay does not obey a simple one-exponential relationship |13|. The fluorescence response functions for donor and acceptor emissions might therefore be more complicated than equations 4 and 7 of paper IV. The theoretical formulations for dipole-dipole energy transfer are summarized in appendix 2. Much more work on model systems like tryptophan-flavin peptides with different end to end distances must be performed in order to get a better insight in the different types of transfer and its inherent mechanisms. Especially at short donor-acceptor distance (<15 Å) not only interactions between two dipoles but also between multipoles should be taken into account.
Perhaps the most important result of paper IV is that the two FAD sites seem to be non-

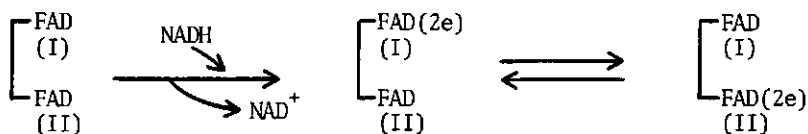
equivalent. This can be concluded from the analysis of the flavin fluorescence decay, which consists of two exponential contributions with almost equal amplitude factors. These two different FAD centers might be connected with two different active sites per molecule of protein. On the other hand the structure and sequence of both subunits seem to be completely identical |14, 15|. The tertiary and quaternary structure of the protein might be responsible for the different micro-environments of the two FAD molecules. The very important question is now: is this just simple coincidence or has this implications for the reaction mechanism? In the enzymatic reaction 2 reducing equivalents are transferred from NADH via FAD to lipoamide and/or from reduced lipoamide via FAD to NAD^+ with the participation of disulfide and thiol groups. In the remaining part of this discussion several points are summarized and some preliminary experiments are reported, the conclusions of which are a logical consequence of paper IV.

Lipoamide dehydrogenase is a constituent of two multi-enzyme complexes, usually abbreviated as pyruvate and α-ketoglutarate dehydrogenase complexes |16|. In the overall reaction pyruvate and α-ketoglutarate are decarboxylated. In the partial reaction the oxidation of dihydrolipoamide, covalently bound to transacetylase, linked to NAD^+ reduction is catalyzed by lipoamide dehydrogenase. When the enzyme is isolated from the complex it is still able to catalyze reversibly the oxidation of derivatives like dihydrolipoamide or reduced lipoic acid linked to NAD^+ reduction. The reaction mechanism is extensively discussed in the literature |17-19|. It is not the purpose to discuss to what kind of mechanism the catalytic action of the enzyme can be classified. One aspect, however, comes forward after reading the literature. In the explanation of the kinetic mechanism the FAD molecules have been considered to be identical and independent. The enzyme can be reduced by its substrate dihydrolipoamide with two reducing equivalents, which are proposed to be distributed over the flavin and disulfide bridge. In a slow side reaction the substrate NADH can reduce the enzyme with four reducing equivalents, the flavin and disulfide bridge being fully reduced. Spectral changes accompany these reactions |17, 20|. The physiological activity seems to be connected with the dimeric form of the enzyme and not with the monomeric form. Dissociation of the enzyme into subunits, induced by increasing concentrations of sodium dodecylsulphate for instance, always leads to loss of activity |21|. When the apoenzyme is made according to the acid ammonium sulphate precipitation method, the structure is monomeric |22, 23|. Recombination with FAD is considered to take place via a multistep process: firstly binding of FAD to monomeric apoenzyme (inactive) followed by dimerisation to active enzyme |24|. These experiments gave unambiguous evidence, that the dimeric enzyme is the active species. The kinetic mechanism was always set up for two sets of independent FAD's and disulfide bridges |18|. Doubt towards this proposal came from the demonstration that the enzyme was able to bind NAD^+ at two different sites - a regulatory site and a catalytic site - susceptible to cooperative interaction |25|. These cooperative effects are indeed demonstrated from kinetic experiments at room temperature but seemed to be absent at elevated temperatures (37 ^{o}C) |19, 26|. Cooperativity indicates that the two active sites are not entirely independent, since binding of the first substrate molecule influences the binding of the second one.

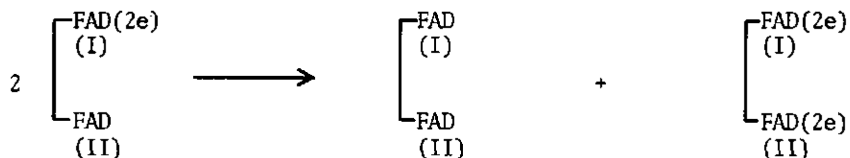
From the observation that the two FAD centers have different micro-environments the following question arises. Is there a preferential substrate binding to one of the two inequivalent FAD sites? Some preliminary experiments were performed to investigate this hypothesis. NADH in (sub)stoechiometric amounts was added anaerobically to the enzyme. NADH forms with lipoamide dehydrogenase a complex with an absorption maximum at about 530 nm. The flavin fluorescence was used as an internal marker for the oxidized state of the flavin. A cross correlation phase and modulation fluorometer operating at 60 Mc was used for several reasons |27|. Fluctuations in the fluorescence parameters like lifetime, quantum yield and polarization were observed when NADH was added to the enzyme. The time course of the fluctuations in lifetime can not be measured with the single photon counting apparatus (cf. paper IV), because with this method statistically meaningful results are obtained after a sampling period, much longer than the duration of the fluctuations. The heterogeneity of the fluorescence can be also detected with this apparatus by measuring simultaneously the two apparent lifetimes via phase shift (τ_p) and modulation depth (τ_m) measurements |27| (see also appendix 1). The large advantage of a cross correlation fluorometer is that changes in lifetimes as a function of time can be measured very easily and that the results are obtained much quicker. The results are summarized as follows:

1) Without addition of NADH the heterogeneity of the flavin fluorescence was confirmed because τ_p and τ_m are different ($\tau_m > \tau_p$) (see appendix 1). The largest difference between τ_p and τ_m is at 4 ^{o}C; it becomes less at higher temperature (37 ^{o}C).
2) Anaerobic addition of a slight (molar) excess of NADH (with respect to FAD) leads only to partial quenching of the flavin fluorescence; the shape of the fluorescence spectrum remaines the same.
3) (Sub)stoechiometric addition of NADH anaerobically results in an initial drop (completed after about one minute) in the values of τ_p, τ_m and the fluorescence intensity (I) followed by a slow increase of the values of these parameters and stabilization after about 15 minutes. These phenomena were observed at 4° and at 22 ^{o}C.
4) (Sub)stoechiometric anaerobic addition of NADH results in a decrease of the degree of the polarization of the fluorescence (p) completed after about one minute. The value of p remaines constant at this lower value.
5) Addition of NAD^+ hardly affected the values of τ_p, τ_m, I and p.

More experiments are needed for a correct explanation, but one might speculate a little bit. Fluorescence can still be observed after addition of a small excess of NADH, which proves that oxidized flavin is present a few seconds after mixing. The results suggest that two electrons are transported within the peptide chain accompanied with structural changes in the protein as proposed in the following scheme:



FAD (I) denotes the FAD characterized by the longest lifetime and largest quantum yield and FAD (II) the flavin component with the opposite properties (see paper IV and appendix 1). FAD(2e) indicates the 2-electron reduced form of the enzyme. The first reaction is fast, the equilibration much slower. In the first reaction FAD (I) is reduced and its fluorescence is lost leaving the much weaker and shorter living fluorescence of FAD (II). Upon redistribution of the 2 electrons towards FAD (II) within the dimeric enzyme the stronger fluorescence of FAD (I) reappears. The drop of p indicates that changes in the micro-environment of one flavin induce changes in the subunit interaction. An intermolecular disproportionation reaction like



can be excluded because the totally reduced form is considered to be nonfluorescent and the reaction should leave p unchanged.
In case of internal transport of electrons, it can be visualized that the enzyme contains two different sites: an input site for NADH and either an output site for lipoamide or a regulatory site for NAD^+. If this electron transport takes place from one flavin to the other a striking similarity with the scheme in fig. 5A of paper I is obvious. In that scheme, which can be considered an elegant artificial side reaction, an electron from one flavin is directly injected into the other one, thus mimicing in a way the possible situation in the dimeric flavoenzyme. In the protein the flavins are possibly not in direct contact, but electrons seem to be transported . Contact between the two S-S bridges could be considered. One argument against a close contact between the two FAD molecules is that p is rather high. This indicates that the two flavins are located at such a distance that no energy transfer among the two flavins occurs, which causes depolarization of the fluorescence |28|. The R_o-value characteristic for transfer between identical flavin molecules is in the order of 14 Å, which means that transfer should be very efficient if the distance between the two flavins is smaller than 14 Å |28|. However if the two aromatic rings are more or less perpendicular oriented, energy transfer is unfavourable, although the two molecules can be positioned at a short distance |28|.
It is needless to say that these arguments are rather speculative and that more experiments need to be performed. These experiments are in progress.

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SAMENVATTING

Dit proefschrift beschrijft enige eigenschappen van flavines, die door absorptie van licht in een hogere aangeslagen toestand zijn gebracht. Met snelle spectroscopische apparatuur is onderzocht hoe en waardoor deze eigenschappen kunnen worden beïnvloed. De verkregen resultaten zijn gepubliceerd in vier artikelen, die in dit proefschrift zijn opgenomen.

Het eerste artikel beschrijft de toepassing van de laserphotolyse techniek op het flavinederivaat 3-methyllumiflavine. Direct na de laserpuls ontstaan drie species, die zich onderscheiden in hun optisch en kinetisch gedrag en in hun afhankelijkheid van de pH van de oplossing. De spectra van twee ervan kunnen worden toegekend aan de absorptiespectra van het flavine in de eerste aangeslagen singlet- en triplet-toestand. Argumenten worden aangevoerd om de derde species toe te schrijven aan een "photodimeer" van twee flavines, dat slechts in de aangeslagen toestand stabiel is.

De tweede publicatie beschrijft de uitkomsten van een ander pulsexperiment. De vrije nucleotiden FMN en FAD zijn bestraald met een gepulsde lichtbron, waarna de fluorescentie als functie van tijd en golflengte is bepaald. De intramoleculaire wisselwerking tussen het adenine- en isoalloxazine-gedeelte van FAD maakt het in theorie mogelijk, dat minstens twee conformaties, de open en de gesloten vorm, kunnen bijdragen aan de totale fluorescentie. Uit de analyse van de fluorescentie-afvalcurven van FMN en FAD in twee exponentiële termen, blijkt eenduidig dat alleen de meer geopende conformatie van FAD fluorescent is. De snelheidsconstante voor "complexvorming" in de aangeslagen toestand van FAD is bij verschillende temperaturen bepaald en hieruit is de activeringsenergie berekend; deze bedraagt 5.5 kcal mol^{-1}.

Een derde artikel bevat informatie, verkregen uit fluorescentie-experimenten, over de localisatie van het FAD en een andere fluorophoor, tryptophaan, in het flavoproteïne lipoamide dehydrogenase. Er kunnen twee verschillende apoenzymen worden bereid, die zich onderscheiden in hun eiwitfluorescenties. In het holoenzym zijn metingen verricht aan stralingsloze energie-overdracht van tryptophaanmoleculen naar het flavine. Op grond van een model kan worden vastgesteld, dat niet alle tryptophanen binnen het eiwitmolecuul hun excitatie-energie afstaan aan het flavine. Uit de experimentele gegevens is de afstand tussen een tryptophaan-flavine paar bepaald; deze bedraagt 13-16 Å.

De vierde publicatie beschrijft een onderzoek naar de tijdsafhankelijkheid van de fluorescentie van de tryptophaan- en flavineresiduen in lipoamide dehydrogenase. De waargenomen fluorescentie-afvalcurven zijn geanalyseerd in twee exponentiële bijdragen. Uit de analyse van de tryptophaan- en gesensibiliseerde flavinefluorescentie blijkt dat niet alle tryptophaanmoleculen bij de energie-overdracht naar het flavine zijn betrokken, zoals ook in het vorige artikel is aangetoond. De afstand tussen een tryptophaan-flavine paar, die uit deze experimenten is bepaald, bedraagt 15 Å. Een nauwkeurige analyse van de fluorescentie-

afvalcurven van de flavines, verkregen na directe aanslag, wijst uit dat de beide flavines niet-equivalente posities in het eiwit innemen. Een vergelijking met de levensduren van de FAD- en FMN-fluorescenties leert, dat bij 4 ^{o}C één van de FAD's in een meer geopende vorm moet voorkomen (langste levensduur), terwijl de andere meer gevouwen is (kortste levensduur).
Aanvullende experimenten zijn in een algemene discussie-sectie vermeld. De resultaten geven aanwijzingen dat electronen binnen de eiwitketen worden getransporteerd, mogelijkerwijze van het ene FAD- naar het andere FAD-molecuul. Dit zou consistent zijn met een verschillende "input- en output-site" voor het enzym.

CURRICULUM VITAE

De auteur behaalde in 1961 het einddiploma gymnasium-B aan het Huygens Lyceum te Voorburg. In hetzelfde jaar begon hij zijn studie chemie aan de Universiteit van Amsterdam. In 1966 werd het kandidaatsexamen f behaald en in 1969 het doctoraalexamen (hoofdvak fysische chemie, bijvakken chemische fysica en theoretische chemie).
Vanaf 1 september 1969 is hij werkzaam bij het Laboratorium voor Biochemie van de Landbouwhogeschool, momenteel als wetenschappelijk medewerker eerste klas.
Het in dit proefschrift beschreven onderzoek heeft hij op drie laboratoria uitgevoerd, t.w. het Laboratorium voor Fysische Chemie van de Universiteit van Amsterdam, het "Centre de Biophysique Moléculaire" van het C.N.R.S. te Orléans Frankrijk en het Laboratorium voor Biochemie van de Landbouwhogeschool.