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Energy transfer in biflavinyl compounds as studied with fluorescence depolarization

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Summary

Light absorption, fluorescence and fluorescence anisotropy measurements were performed on two different, dimeric flavin models and on the parent isoalloxazine derivative, either dissolved in chloroform or in the rigid matrix polymethylmetacrylate (PMMA). The optical results in fluid solution and the fluorescence depolarization in PMMA could be well explained by a different intramolecular equilibrium orientation of the flavin constituents. The rate constant for radiationless energy transfer among the flavins within the dimers followed directly from an analysis of the anisotropy decay.

energy transfer; flavin compounds; fluorescence depolarization studies

Introduction

Energy transfer processes are of vital importance in living matter, the most outstanding example being the key function of chlorophyll aggregates in light energy conversion. Flavins belong to another class of photobiologically important chromophores. The pigments are responsable for light transformation processes like phototropism and (blue light) photoreception [1]. Flavins and especially deazaflavins are also powerful photocatalysts [2,3]. In the presence of an electron donor light-excited (deaza) flavins can reduce redox compounds of low potential. If the reducable redox

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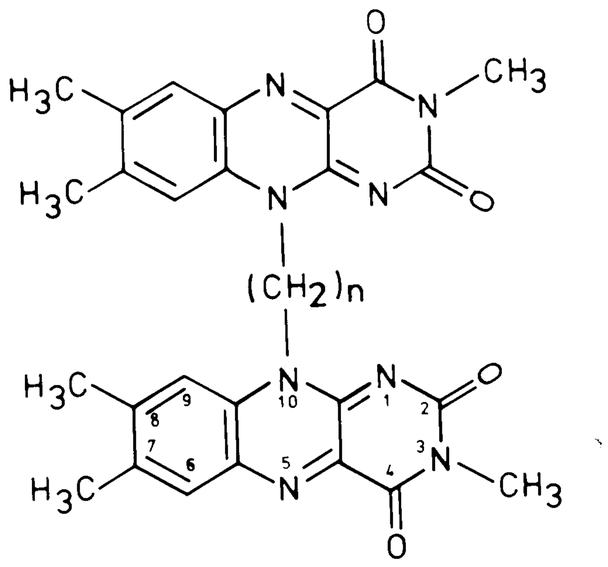
carrier is a flavoprotein it is obvious that interflavin contact is essential for efficient photoreduction. In order to investigate proximity relationships between flavins we have synthesized two dimeric flavin models, in which the two chromophores are separated by an alkyl chain consisting of three respectively six methylene residues (cf. Scheme I).



Scheme I. Structure of flavin dimers. $n = 3$: $Fl(CH_2)_3Fl$; $n = 6$: $Fl(CH_2)_6Fl$.

The strong fluorescence of the compounds invites the use of fluorescence techniques to study flavin–flavin interaction. In this paper the fluorescence properties of the flavin dimers are compared to those of flavin monomer under identical conditions. Two solvent systems were employed. Diluted solutions of the flavin compounds in chloroform yield an extremely flexible system, in which intramolecular collisional contact is possible diminishing both the efficiency and the lifetime of the flavin fluorescence in the dimers. Diluted solutions of flavin compounds in polymethylmetacrylate (perspex, PMMA) comprise the other system, in which the viscosity is so high, that the fluorophores are rigidly held in the matrix during the lifetime of the excited singlet state. The sole source of fluorescence depolarization arises then from intramolecular energy transfer between the two flavins. The amount of depolarization is dependent on the relative orientation of the two flavins and the data are used to estimate the relative angles between the first excited singlet transition moments of both flavin constituents. We were also able to resolve the energy transfer process in time by measuring the time-dependent fluorescence anisotropy.

Materials and Methods

Materials

The synthesis of tri- and hexamethylene-bis-10,10′-(7,8-dimethylisoalloxazine) has been described in detail by Leonard and Lambert [4]. The compounds were converted into the trimethylated derivatives by *N*(3)-methylation of the isoalloxazine parts (see Ref. 5 and references cited therein). The compounds, abbreviated as $Fl(CH_2)_3Fl$ and $Fl(CH_2)_6Fl$, were purified by column chromatography on silica gel (Kieselgel Mallinckrodt, Serva, Heidelberg) eluted with $CHCl_3$ containing 0.5–1%

CH_3OH. Purification was tested on TLC (Bakerflex silica gel IB_2, J.T. Baker) with *n*-butanol/glacial acetic acid/water (7 : 2 : 1, v/v/v) or acetonitrile as eluents and was verified by mass spectrometry carried out at the Department of Organic Chemistry, Agricultural University, Wageningen. 3-Methyllumiflavin (MLF) was synthesized as described in reference [5]. The incorporation of flavin compounds in polymethylmetacrylate has been detailed elsewhere [6].

Methods

Absorption and fluorescence spectra and polarizations were obtained as previously described [7]. Fluorescence lifetimes were measured on a 60 MHz phase fluorimeter [8] or with an argon laser-single photon counting system ($\lambda_{exc} = 458$ nm, $\lambda_{em} = 531$ nm), extensively described elsewhere [9–11]. The measurements of the decay of fluorescence anisotropy have also been detailed [12]. However, since the anisotropy decay occurs in the subnanosecond time region deconvolution procedures are required, which will be shortly summarized.

(1) The vertical and horizontal polarization components $I_{\parallel}(t)$ and $I_{\perp}(t)$ are measured under identical conditions and are combined to the following decay curves:

$$s(t) = I(t) + 2I_{\perp}(t) \text{ and } d(t) = I_{\parallel}(t) - I_{\perp}(t) \qquad \text{(1a and b)}$$

(2) $s(t)$ and $d(t)$ can be considered as convolution products of the excitation function ($g(t)$), measured separately, and the decay function $S(t)$ and $D(t)$:

$$s(t) = \int_0^t g(t')S(t-t')\,dt' \text{ and } d(t) = \int_0^t g(t')D(t-t')\,dt' \qquad \text{(2a and b)}$$

(3) The anisotropy is defined as $r(t) = D(t)/S(t)$ and can be obtained from the convolution relation:

$$d(t) = \int_0^t g(t')r(t-t')S(t-t')\,dt' \qquad (3)$$

In Eqn. 3 we firstly determined $S(t)$ by fitting the $s(t)$ data to a single exponential function. Using this $S(t)$ we fitted the $d(t)$ data to the convolution product of $g(t)$ and $r(t)$ $S(t)$ to yield the best fit parameters for the selected model of $r(t)$.

(4) To achieve this parameter fitting we used the non-linear least-squares algorithm as outlined by Grinvald and Steinberg [13].

(5) As will be evident from the results the model selected for $r(t)$ is of the form:

$$r(t) = (r_0 - r_\infty)e^{-2k_t t} + r_\infty \qquad (4)$$

Eqn. 4 consists of a time-dependent part with rate constant k_t and a time-independent part r_∞.

Tanaka and Mataga [14] have considered time-dependent photoselection in weakly interacting dimers composed of identical monomers A and B. Since the latter system is similar to the biflavinyl compounds it is helpful to summarize the expressions derived by Tanaka and Mataga for steady state and time-dependent anisotropies [14]:

$$\langle r \rangle = \frac{3}{5(k_f + 2k_t)}\left[k_f(\cos^2\alpha - 1/3) + k_t(\cos^2\alpha + \cos^2\beta - 2/3)\right] \qquad (5)$$

$$r(t) = \tfrac{3}{10}\left[\left(\cos^2\alpha + \cos^2\beta - 2/3\right) + \left(\cos^2\alpha - \cos^2\beta\right)e^{-2k_t t}\right] \quad (6)$$

In Eqns. 5 and 6 α is the angle between absorption and emission transition moments in the monomers A and B; in the equations of Tanaka and Mataga we have assumed that $\beta_1 = \beta_2 \equiv \beta$ (β_1 and β_2 are undistinguishable), β_1 and β_2 are the angles between the absorption moment in A and the emission moment in B and vice versa, respectively; $k_f = 1/\tau_f$ is the rate constant of fluorescence, identical for both monomers; k_t is the rate constant for radiationless energy transfer via Förster's mechanism [15]. This exchange of energy takes place in the excited singlet states of A and B; the rate constants for transfer A to B and from B to A are assumed identical. Eqn. 6 is of the same form as Eqn. 4 and least squares analysis thus provides both the transfer rate constant and orientational information.

It should be noted that at $t = 0$ we have:

$$r_0 = \tfrac{1}{5}(3\cos^2\alpha - 1) \quad (7)$$

and at $t = \infty$:

$$r_\infty = \tfrac{3}{10}\left(\cos^2\alpha + \cos^2\beta - 2/3\right) \quad (8)$$

Eqn. 8 is identical to Eqn. 5 if the energy transfer is much faster than the fluorescence process ($k_t \gg k_f$). This is exactly the case in the flavin dimers.

Results and Discussion

The solubility of the dimer strongly limited the choice of solvent. Chloroform proved to be an appropriate medium and we used this solvent for spectroscopic characterization. Absorption spectra of the biflavinyl compounds are slightly different from that of monomer flavin. Fig. 1 shows absorption difference spectra for illustration. It is evident that the excited state vibrational levels are slightly perturbed by the mutual interaction. If attention is focussed on the first absorption band (400–500 nm), it is clear that the intramolecular perturbation induces hypochromism in $Fl(CH_2)_3Fl$, but a small hyperchromism in $Fl(CH_2)_6Fl$. Since hypochromism and hyperchromism depend very much on the equilibrium configuration [16], these absorption difference spectra indicate geometric variations among the two dimers.

The fluorescence spectrum of $Fl(CH_2)_3Fl$ exhibits a hypsochromism of 2 nm as compared to MLF and $Fl(CH_2)_6Fl$ (Fig. 2). The similar fluorescence spectral distribution of monomer and dimers indicates nearly equal Franck–Condon factors. Table I compiles the fluorescence parameters. $Fl(CH_2)_3Fl$ has the shortest lifetime and smallest fluorescence efficiency. Two types of interaction can explain shortening of the lifetime. Energy transfer is one determining factor, accompanied with the creation of additional radiationless deactivation channels diminishing both lifetimes and quantum efficiencies. The other process is quenching due to intramolecular collisions. This mutual quenching can occur in a fluid solvent, since in the composite system the two isoalloxazine moieties are flexibly connected. Both types of quench-

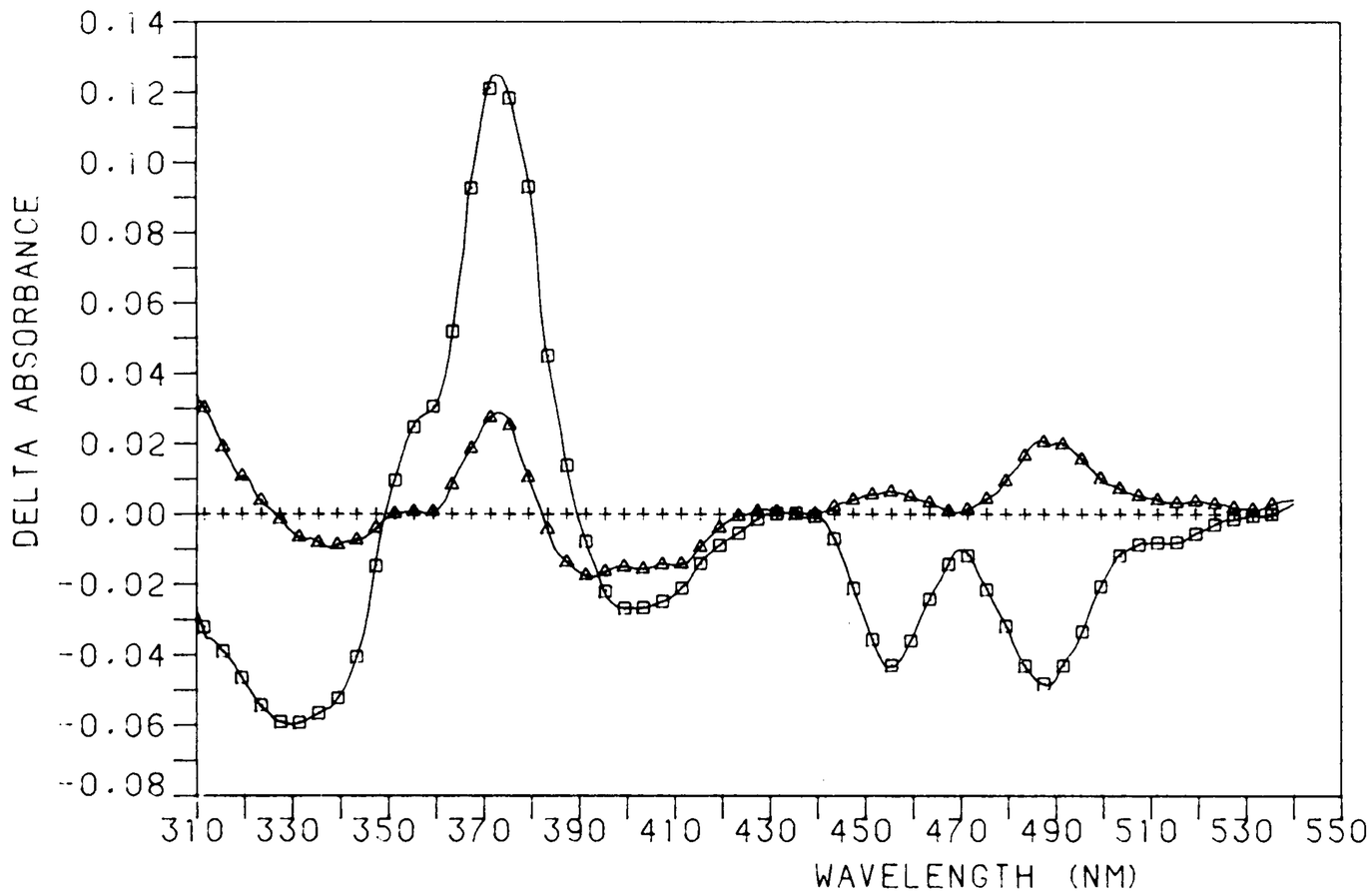


Fig. 1. Absorption difference spectra of $Fl(CH_2)_6Fl$ (△) and $Fl(CH_2)_3Fl$ (□) in chloroform against a matched (at 440 nm) MLF/$CHCl_3$ solution ($A^{1cm}_{440nm} = 0.585$) at 20°C. The baseline (+) was linearized with two identical MLF/$CHCl_3$ solutions in sample and reference compartments.

ing can be expected to be more efficient in $Fl(CH_2)_3Fl$ because of the shortest distance.

To eliminate the component due to dynamic quenching the compounds in PMMA can be studied making the fluorescence anisotropy data of the biflavinyl

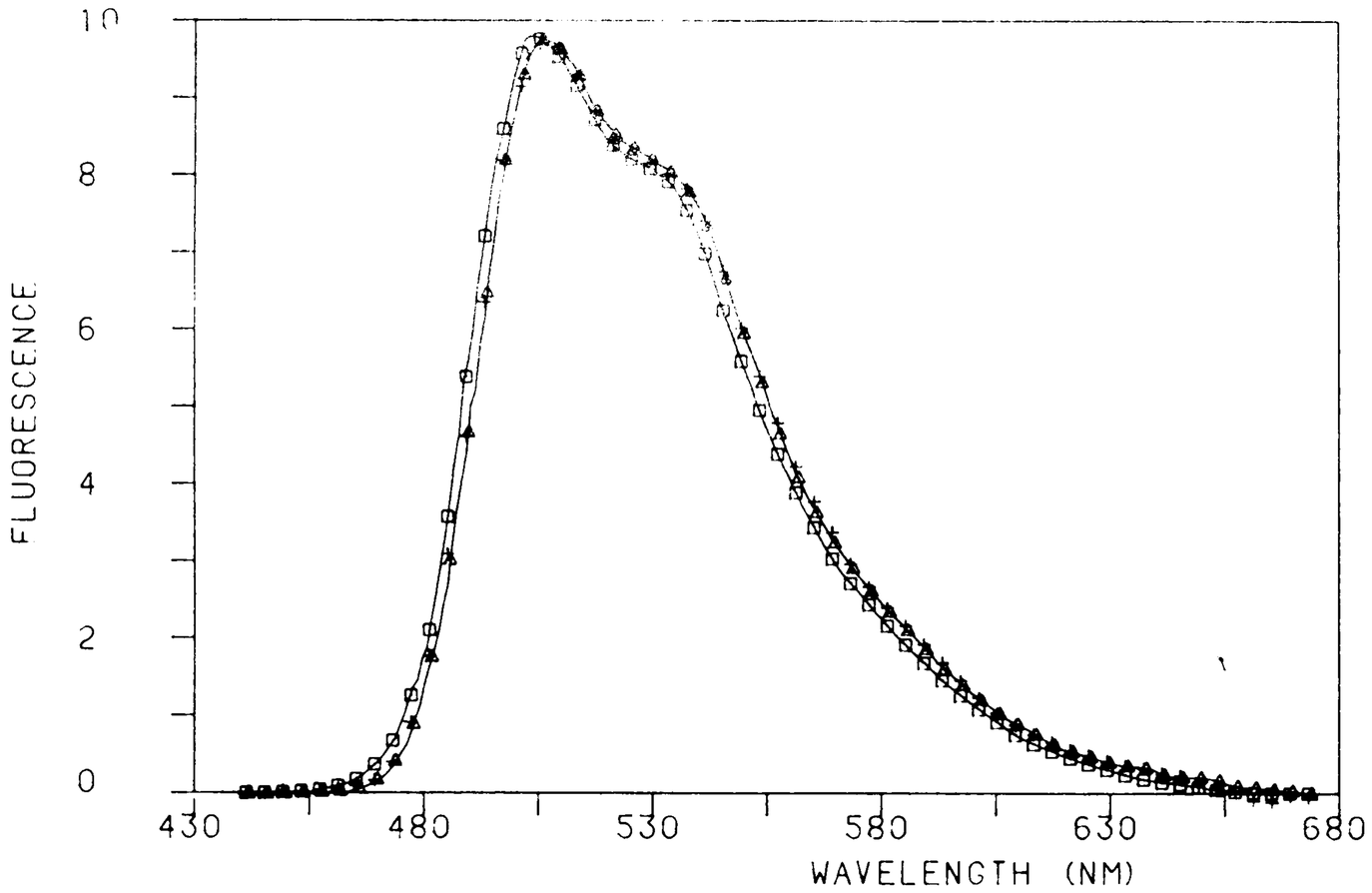


Fig. 2. Peak normalized fluorescence spectra of $Fl(CH_2)_6Fl$ (△), $Fl(CH_2)_3Fl$ (□) and MLF (+) in chloroform at 20°C. The spectrum of $Fl(CH_2)_3Fl$ is shifted hypsochromically relative to the other overlapping spectra.

TABLE I

FLUORESCENCE LIFETIMES AND RELATIVE QUANTUM EFFICIENCIES OF BIFLAVINYL COMPOUNDS AND MLF IN CHLOROFORM AT 20°C [a]

Compound	τ(ns)	τ/τ_0	q/q_0
MLF	6.90	1.0	1.0
$Fl(CH_2)_3Fl$	4.85	0.70	0.71
$Fl(CH_2)_6Fl$	6.13	0.89	0.85

[a] $\lambda_{exc} = 440$ nm, $\lambda_{em} > 500$ nm

compounds more interesting, since the only source of depolarization must be found in energy exchange among both flavin constituents. In Fig. 3 we have plotted the experimental anisotropy as function of time for the three different compounds. Time zero is coincident with the maximum of the impulse response (Fig. 4A). The anisotropies at 10 ns after the pulse coincide with the steady state values. Furthermore, a rapid depolarization can be discerned in the dimers. The characteristic relaxation time is of the same order as the laser pulse width. Therefore the data need to be deconvoluted.

Following the procedure as outlined in Methods the total decay and the difference between the polarized components have been analyzed separately. The total sum curve $s(t)$ provides the fluorescence lifetime (τ_f), while the difference curve $d(t)$ can be analyzed to yield r_0, r_∞ and the transfer rate constant k_t. Graphs are shown in Fig. 4A–C and the results have been gathered in Table II. Fig. 4A shows that the

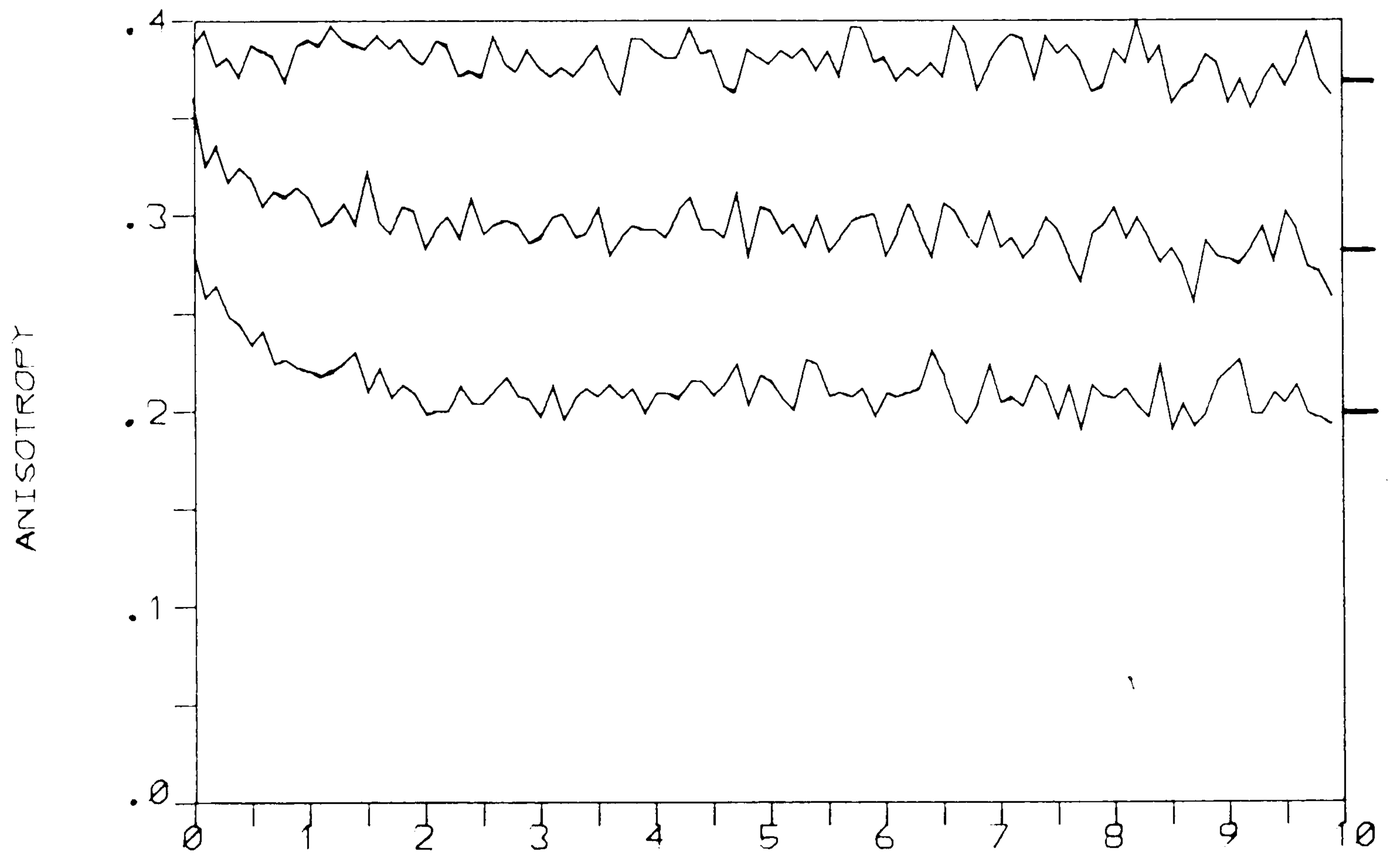


Fig. 3. Time dependent anisotropies of $Fl(CH_2)_3Fl$, $Fl(CH_2)_6Fl$ and MLF in PMMA. Time zero is equivalent to the maximum of the laser pulse. Steady state anisotropies of the three compounds are marked at the right.

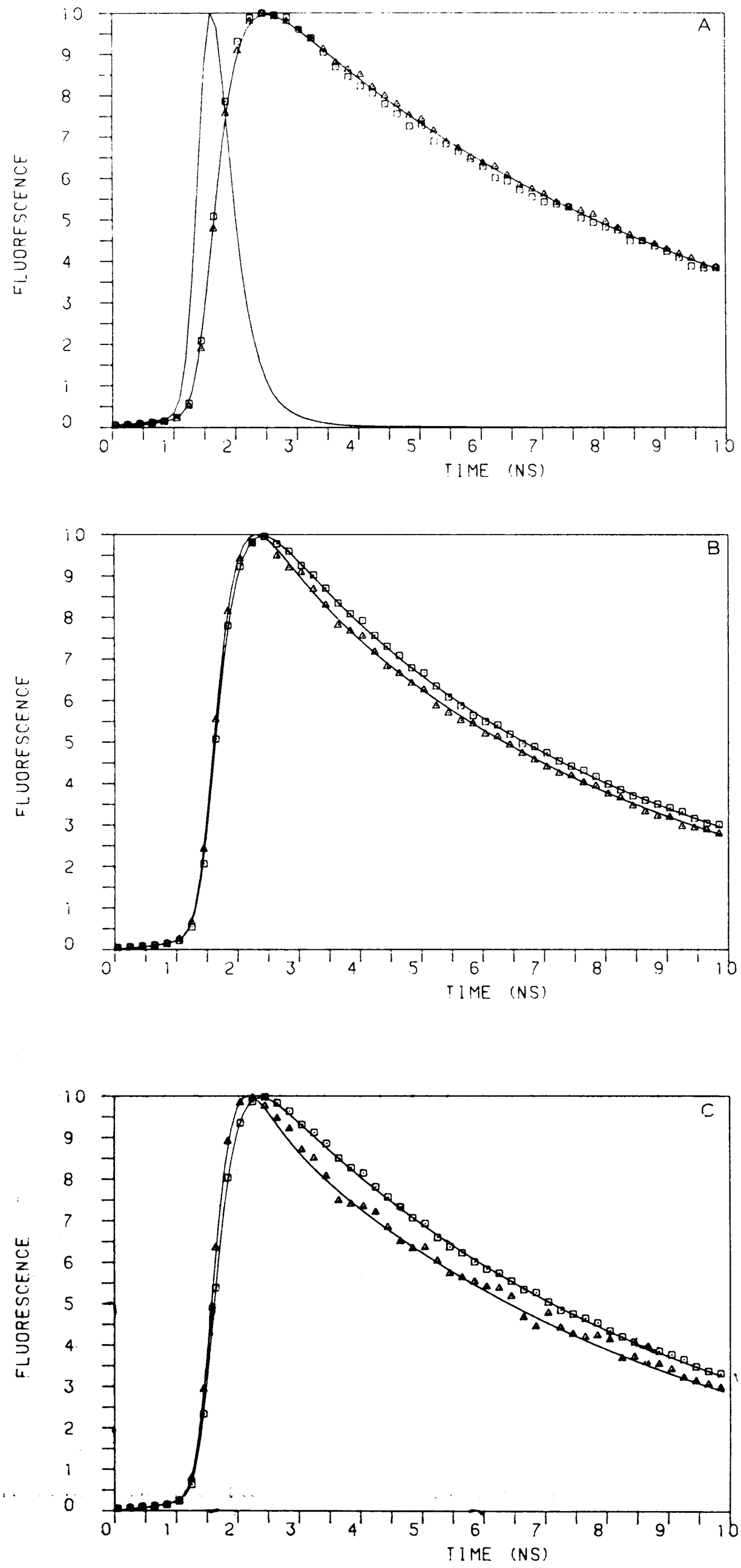


Fig. 4. Sum (⊡) and difference (◬) decay curves and their fits (———) of MLF (A), Fl$(CH_2)_3$Fl (B) and Fl$(CH_2)_6$Fl (C). All curves are normalized to their peak values. The parameters are listed in Table II. The laser pulse is only shown in the top panel.

TABLE II

ANISOTROPY CHARACTERISTICS OF FLAVIN MONOMER AND DIMERS IN PMMA [a]

Compound	τ_f (ns)	k_t (10^9 s^{-1})	r_0	r_∞ [b]	$\langle r \rangle$ [c]	α	β
MLF	7.4	–	0.38	0.37	0.367	11°	–
$Fl(CH_2)_3Fl$	5.9	0.9	0.38	0.27	0.281	11°	39°
$Fl(CH_2)_6Fl$	6.4	1.6	0.37	0.20	0.195	13°	52°

[a] See Method section for explanation of symbols.
[b] Estimated at 10 ns after the pulse.
[c] Steady-state fluorescence anisotropy, $\lambda_{exc} = 458$ nm, $\lambda_{em} > 500$ nm.

normalized $d(t)$ and $s(t)$ curves of the monomer can be superimposed, both yielding the same fluorescence time constant. The $d(t)$ and $s(t)$ curves of both dimers have different shapes owing to the time dependence of the depolarization. Noteworthy are the high r_0 values obtained from the analysis; also the transfer rate constant k_t is an order of magnitude larger than the rate constant of fluorescence $k_f (= 1/\tau_f)$. The latter observation suggests that several transfers are possible during the much longer excited state lifetime.

The orientation angles α and β can be obtained straightforwardly by substituting the values of r_0 and r_∞ in Eqns. 7 and 8. Owing to the exceptionally high r_0 (only slightly less than the limiting value 0.40) the angle α between absorption and emission transition moments in a single molecule is small and is similar for all three compounds (Table II). The significance of the angle β should be considered with care, since a distribution of intrachromophoric distances and orientations prevails for the dimers dissolved in PMMA; β is in a strict sense not a single angle in space, but rather represents a distribution of β-values. The contribution of each β will depend upon the transfer rate constant k_t. k_t therefore represents an ensemble average, not a single rate constant as assumed in Eqn. 4 for an unique intrachromophoric configuration. Possibly, owing to the deconvolution procedure, no deviation from Eqn. 4 could be detected. Nonetheless, the angle β between transition moments of two flavins in the dimer that follows from Eqn. 8 is distinctly different for both dimers and reflects the different relative orientation of both isoalloxazine planes.

The small spectral alterations, most markedly for the shortest dimer $Fl(CH_2)_3Fl$ as shown in Figs. 1 and 2, limits the applicability of Förster's mechanism [15], because stronger interaction has to be taken into account. Another indication is that the fluorescence lifetimes in PMMA are different for the monomer and both dimers, whereas in the Förster limit the lifetime should not change in case of energy transfer between like molecules. The lifetime shortening is probably due to additional radiationless deactivation pathways in the two dimers as compared to the monomer. The Förster formulation yields a transfer rate, which is inversely proportional to the sixth power of the intrachromophoric distance R. Stronger coupling gives a transfer rate which varies as $1/R^3$, thus resulting in a longer range interaction. The stronger

coupling limit together with the concept of the transfer rate constant as an ensemble average detracts from the significance of the different k_t values found for the two dimers.

Summarizing, both optical and fluorescent properties of these dimers have demonstrated that the mutual orientation of the two isoalloxazine rings is different in the two cases studied. More specifically, the depolarization of the fluorescence is due to exchange of energy among the flavin constituents. This observation may provide an alternative interpretation of the polarized emission properties of oligomeric flavoproteins [17]. The methodology presented here can very well be applied to other light energy transducing systems like the chlorophylls.

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