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PICOSECOND FLUORESCENCE DYNAMICS OF REDUCED FLAVINS

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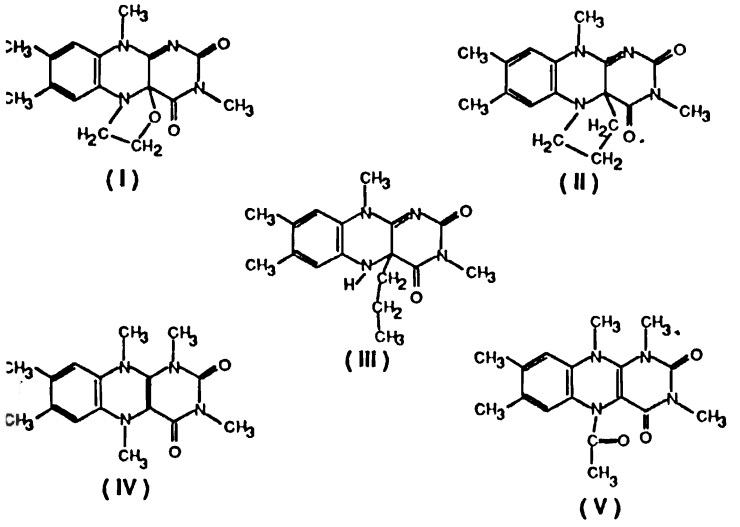
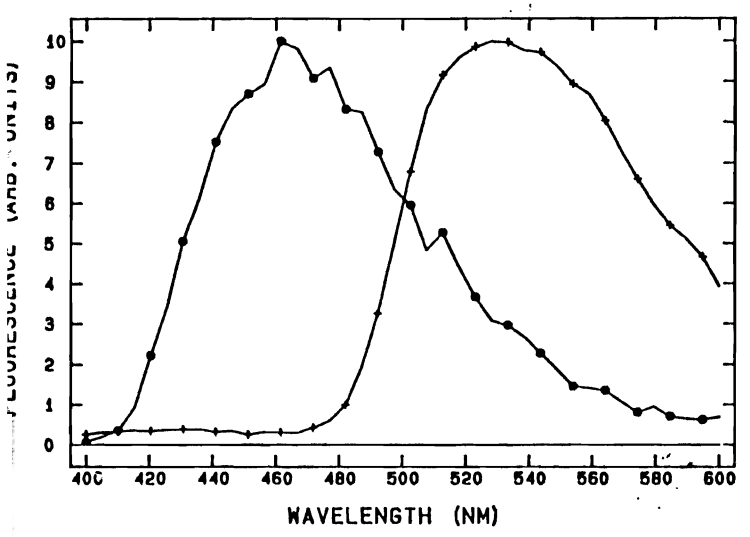
Introduction

Reduced flavins and flavoproteins are potentially fluorescent (1). The fluorescence quantum efficiency and spectral distribution strongly depend on an interplay of factors, varying from viscosity and polarity of the solvent to the nature and position of substituents. There are two types of reduced flavin compounds: the 1,5-dihydroflavins and the 4a,5-dihydroflavins. The excited flavins with oxygen added to the C_{4a}-position (peroxide- and hydroxy-substituted) play an important role as fluorescent transients in the bacterial luciferase reaction (2-4). Protein-bound reduced flavins exhibit, in some cases, relatively intense fluorescence, which is rather long-lived as compared to the fluorescence of the flavin in the oxidized state (3-5). On the other hand, the emission of reduced flavin compounds in fluid solvents is very weak (1), with fluorescence lifetimes in the subnanosecond time domain (5). These observations strongly suggest that rigidity of both solvent environment and molecular structure determines the appearance of fluorescence, thus also determining the balance between radiative and radiationless decay processes.

The progress that has been made during the last decade in obtaining fluorescence decay profiles, with picosecond resolution and high sensitivity, has prompted us to reinvestigate the dynamic fluorescence properties of reduced flavin compounds. Instead of an analysis in discrete fluorescence lifetimes, the results were analysed using lifetime distributions. The results of the experiments and the implications in relation to a physical model will be briefly described.



'igure 1. Structure of reduced flavins.



igure 2. Fluorescence spectra of compounds I (+) and IV (o).

Materials and Methods

The flavin compounds used are listed in Figure 1. Their synthesis and nomenclature have been described previously (1,6). Compounds I-III are 4a,5-dihydrolumiflavins, derivatives IV and V are 1,5-dihydrolumiflavins. The reduced flavins were dissolved in ethanol. The time-correlated single photon counting system with the frequency-doubled, cavity-dumped dye laser, pumped by a mode-locked Nd-YAG laser, as source of excitation, has been described elsewhere (7). The excitation wavelength was 375 nm, and monochromatic emission was detected through a polarizer at magic angle at 520 nm (I), 550 nm (II), 510 nm (III), 465 nm (IV) and 450 nm (V). The temperature was always 20 °C. Decay curves were collected up to 10^5 counts in the peak channel. The analysis in fluorescence lifetime distributions has been accomplished with the exponential series method provided by the program CONTIN (8). Up to 50 points equally spaced in logτ intervals between 0.010 and 10.0 ns were used for the reconstruction of the lifetime distribution pattern.

Results and Discussion

Corrected fluorescence spectra of flavins I and IV, normalized to the emission maximum, are presented in Figure 2 in order to emphasize the different emissions of the two classes of dihydroflavins. The fluorescence yield of compound I is by far the largest, as can be concluded from the collected data in Table 1 (the fluorescence efficiency of I is set equal to 1.0). The comparison between the 4a,5-bridged compounds I and II is quite interesting. It must be concluded that addition of oxygen at C_{4a}, in combination with the more rigid structure as imposed by the bridge, leads to the strongest fluorescence of these reduced flavins. This empiric finding is in agreement with the significant fluorescence in C_{4a}-oxygen adducts in luciferase reaction intermediates.

Table 1. Fluorescence parameters of reduced flavins in ethanol.

Compound	λ_{max} (nm)	Fluorescence efficiency	Lifetime (ps)	Width (ps)	Relative amplitude
I	532	1.0	220	200	1.00
			5300	1400	0.33
II	560	0.006	14	6	1.00
III	530	0.123	60	40	1.00
			5700	1400	0.01
IV	467	0.111	17	12	1.00
			76	23	0.04
V	469	0.256	28	20	1.00
			900	300	0.01

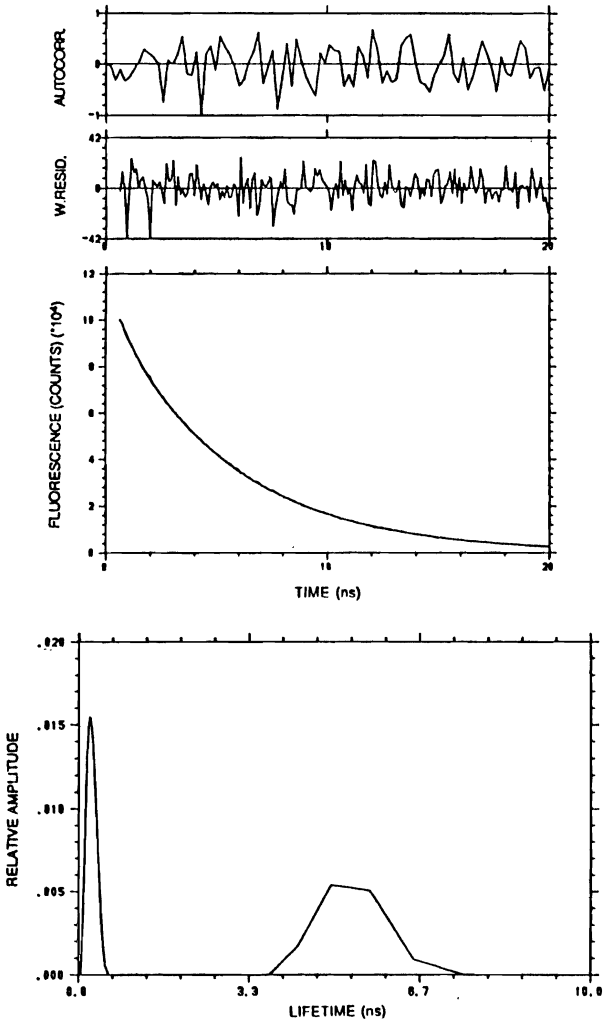


Figure 3. Example of analysis of fluorescence decay of compound I with lifetime distributions. Top: experimental and fitted fluorescence with statistics. Bottom: reconstruction of distribution.

An example of a fluorescence decay analysis using a lifetime distribution is presented in Figure 3 for compound I. The analysis reveals a bimodal distribution with one peak centered at 220 ps and a broad distribution, centered at 5.3 ns. The area under the curves reflects the steady-state fluorescence intensity, which means that the broad band at 5.3 ns represents a considerable amount of fluorescence in agreement with the fact that compound I is by far the strongest emitter. In Table 1, the results of the lifetime distributions have been collected. Compounds II-V are characterized by narrow picosecond distributions, and for flavins III, IV and V, there is, in addition, a weak distribution of longer lifetimes (Table 1). Compound II has the shortest fluorescence (14 ps), in agreement with the very weak emission, while there is a single lifetime distribution.

The central ring of reduced flavins is essentially nonplanar. The presence of multiple, nonplanar conformations of the flavin, already in the ground state, has important consequences for the photophysical properties when the time scale of the molecular dynamics is considered. Collective molecular vibrations are believed to occur in the frequency range of tens of GHz (9), which corresponds to a subpicosecond "lifetime" of a particular configuration. For reduced flavins, a simple model can be proposed, which may explain the fluorescence results. Let us assume that the flavin can adopt two conformations, which can interconvert. One conformation is more planar, and gives rise to relatively strong, nanosecond fluorescence. The other conformation is bent (distorted) and will lead to weak fluorescence of picosecond duration. This situation corresponds with an interconverting two-state system, where each state is characterized by its own fluorescence decay rate (10). When the rates of interconversion are much larger than the fluorescence decay rates, the fluorescence decay is a single exponential, and the decay rate is a kind of harmonic mean of the individual fluorescence rate constants. On the other hand, when the interconversion is much slower than the fluorescence, a biexponential fluorescence decay would be observed with fluorescence lifetimes characteristic of the two states. A fluorescence lifetime distribution would account for a distribution of interconversion rates. Such a situation implies the presence of various substates superimposed on the two states, and consequently, a bimodal distribution would result in two average lifetimes. The fluorescence kinetics of an interconverting two-state system has been treated in detail very recently (11). Within the limits of the model all rate constants involved can be determined from the two average lifetimes. The quantification for reduced flavins will be reported elsewhere.

Conclusions

The fluorescence decay of reduced flavin model compounds in ethanolic solution is dominated by lifetime components of picosecond duration It must therefore be concluded that ultra fast

collective vibrations are responsible for the fact that reduced flavin possesses multiple, interconverting conformational states. Upon excitation, most states will return to the ground state via radiationless pathways, which explains the very short fluorescence lifetimes observed in the majority of reduced flavins. The presence of multiple conformers, already in the ground state, rationalizes the analysis into lifetime distributions.

Acknowledgement

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References

1. Ghisla, S., V. Massey, J.M. Lhoste, S.G. Mayhew. 1974. Biochemistry 13, 589.

2. Kurfürst, M., S. Ghisla, J.W. Hastings. 1984. Proc. Natl. Acad. Sci. USA 81, 2990.

3. Lee, J., D.J. O'Kane, B.G. Gibson. 1988. Biochemistry 127, 4862.

4. Lee, J., D.J. O'Kane, B.G. Gibson. 1988. Biochemistry 28, 4263.

5. Visser, A.J.W.G. , S. Ghisla, V. Massey, F. Müller, C. Veeger. 1979. Eur. J. Biochem. 101, 13.

6. Ghisla, S., U. Hartmann, P. Hemmerich, F. Müller. 1973. Justus Liebigs Ann. Chem., 1388.

7. Lee, J., D.J. O'Kane, B.G. Gibson. 1989. Biophys. Chem. 33, 99.

8. Provencher, S.W. 1982. Comput. Phys. Commun. 27, 229.

9. Platenkamp, R.J., H.D. van Osnabrugge, A.J.W.G. Visser. 1980. Chem. Phys. Lett. 72, 104.

10. Alcala, J.R., E. Gratton, F.G. Prendergast. 1987. Biophys. J. 51, 597.

11. Ruggiero, A.J., D.C. Todd, G.R. Fleming. 1990. J. Am. Chem. Soc. 112, 1003.