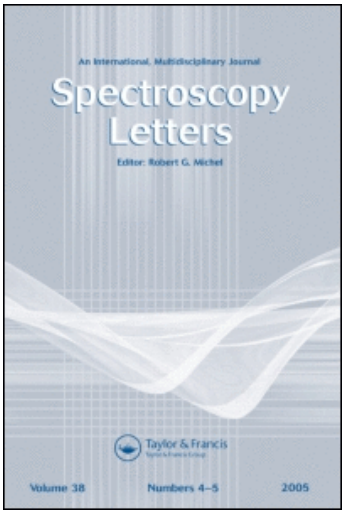

Spectroscopy Letters
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597299

Unusual Properties of Amphiflavins in a Binary Solvent

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To cite this Article Wieczorek, Zbigniew J. and Drabent, Regina(1990) 'Unusual Properties of Amphiflavins in a Binary Solvent', Spectroscopy Letters, 23: 1, 13 — 27
To link to this Article: DOI: 10.1080/00387019008054033
URL: http://dx.doi.org/10.1080/00387019008054033

SPECTROSCOPY LETTERS, 23(1), 13-27 (1990)

UNUSUAL PROPERTIES OF AMPHIFLAVINS IN A BINARY SOLVENT

Key words: amphiflavins, selforganization, absorption and fluorescence spectra of flavins

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SUMMARY

The interaction of amphiflavins in organic solvents and a binary (ethanol/water) solvent has been studied. The absorption spectra, steady state and phase sensitive fluorescence spectra, lifetimes, anisotropy and quantum yield of fluorescence have been measured. In a binary solvent the interaction between the amphiflavin molecules and the microenvironment of different polarity leads to the selforganization of amphiflavins. As a result organized structures of amphiflavin molecules appear.

INTRODUCTION

In natural biological structures flavins are bound to flavoproteins or they occur in the free state. Both biochemical properties and the biological activity of flavins are significantly affected by physical-chemical

properties of their microenvironment. Flavins usually have two characteristic absorption bands (at about 330nm - the S2 peak and 440nm - the S1 peak), which are very sensitive to solvent polarity. Studies on the solvent effect on absorption spectra had been undertaken in the 1960's [1-4]. By comparing the absorption spectra measured in polar and non-polar solvents it can be generally stated that in non-polar solvents the S1 band exhibits a slight hypsochromic effect and its vibronic structure is revealed, whereas the S2 band has a high hypsochromic effect (20-40nm) and exhibits an insignificant hypochromic effect only. For numerous flavoproteins absorption spectra are almost identical with flavin spectra in non-polar solvents. Thus it can be assumed that the active centre of these flavins is hydrophobic [5,6]. Similar spectra are obtained for D-amino acid oxidase [7] and lactose oxidase [8] after binding inhibitors. In all these cases, however, the hypsochromic effect of the S2 band is not as strong as in the case of flavins in non-polar solvents. The formation of hydrogen bonds with solvent molecules or with proteins can also be observed on the basis of absorption spectra of flavins [9-11]. Both theoretical calculations and the results of experiments show the disappearance of the S1 band fine structure and a shift of both bands (especially S2) towards longer wavelengths when compared to spectra in non-polar solvents [12].

The solvent effect on a fluorescence spectrum is most often correlated with the changes of the absorption spectrum. Absorption band shifts towards longer wavelengths usually result in fluorescence shifts in the same direction. The structuralization of the S1 band is most often accompanied by structuralization detected in the fluorescence spectrum. Also, when moving from polar to non-polar solvents a considerable growth of quantum

yield and fluorescence decay time is observed. The formation of hydrogen bonds with solvent molecules or with proteins causes, as in absorption, the disappearance of the fluorescence spectrum vibronic structure as well as a decrease of quantum yield.

Very interesting results of studies on amphiphilic derivatives of flavins have been presented by Shinkai [13,14]. He obtained the fine structure of the S1 band of 3-methyl-10-dodecylisoalloxazine in aqueous solution which had never been observed for any other flavins. This phenomenon has been ascribed by the authors to be the result of the formation of stacking aggregates of this compound, and hydrophobic interaction of long hydrocarbon chains is suggested to be the force leading to aggregation.

The aim of the present work was to investigate the interaction of selected amphiflavins in a binary solvent of various polarity. Molecular absorption and fluorescence spectroscopy have been applied.

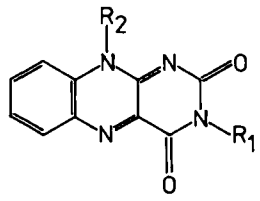
EXPERIMENTAL

The following isoalloxazine derivatives have been used for the experiment (Fig. 1):

(1) 10-dodecylisoalloxazine (DIA)
(2) 10-octadecylisoalloxazine (OIA)
(3) 3-methyl-10-dodecylisoalloxazine (MDIA)

DIA and MDIA were prepared by Dr. P.F. Heelis (School of Natural Science, North E. Wales, Great Britain), OIA was synthesised by Dr. Z. Kazimierczuk (Department of Biophysics, University of Warsaw) according to the method described by Shinkai et al. [14].

The applied solvents (carbon tetrachloride, chloroform, ethanol) were spectrally pure or pure for analysis and were obtained from POCh (Poland). The experiments were carried out at 10μM amphiflavin



(1) R_1=H, R_2=n-$C_{12}H_{25}$
(2) R_1=H, R_2=n-$C_{18}H_{37}$
(3) R_1=CH_3, R_2=n-$C_{12}H_{25}$

FIG. 1. Formulae of amphiflavins used:
(1) - 10-dodecylisoalloxazine (DIA),
(2) - 10-octadecylisoalloxazine (OIA),
(3) - 3-methyl-10-dodecylisoalloxazine (MDIA).

concentrations. Amphiflavin solutions in binary solvents were obtained by mixing water with ethanol dye solutions. The maximum dye concentration in ethanol at room temperature was approx. equal to 0.2mM for DIA and 0.1mM for OIA and MDIA. This limited the possible range of concentrations in binary solvents. The investigated amphiflavins were found to be insoluble in water.

Absorption spectra were measured with the PMQ-II spectrophotometer with a Zeiss MM-12Q double monochromator (FRG) and with the M-40 spectrophotometer (C. Zeiss-Jena, GDR). Measurements were carried out at room temperature. Fluorescence spectra, the quantum yield and anisotropy of fluorescence were measured with a typical arrangement set up in our laboratory. The quantum yields have been determined using fluorescein (2×10^{-6}M concentration) in 0.1N NaOH. The fluorescent standard has a quantum yield of 0.92 [15]. Fluorescence decay times and phase-sensitive fluorescence spectra were measured with a 12MHz phase fluorometer [16,17]. Fluorescence decay curves were obtained using a pulse fluorometer [18].

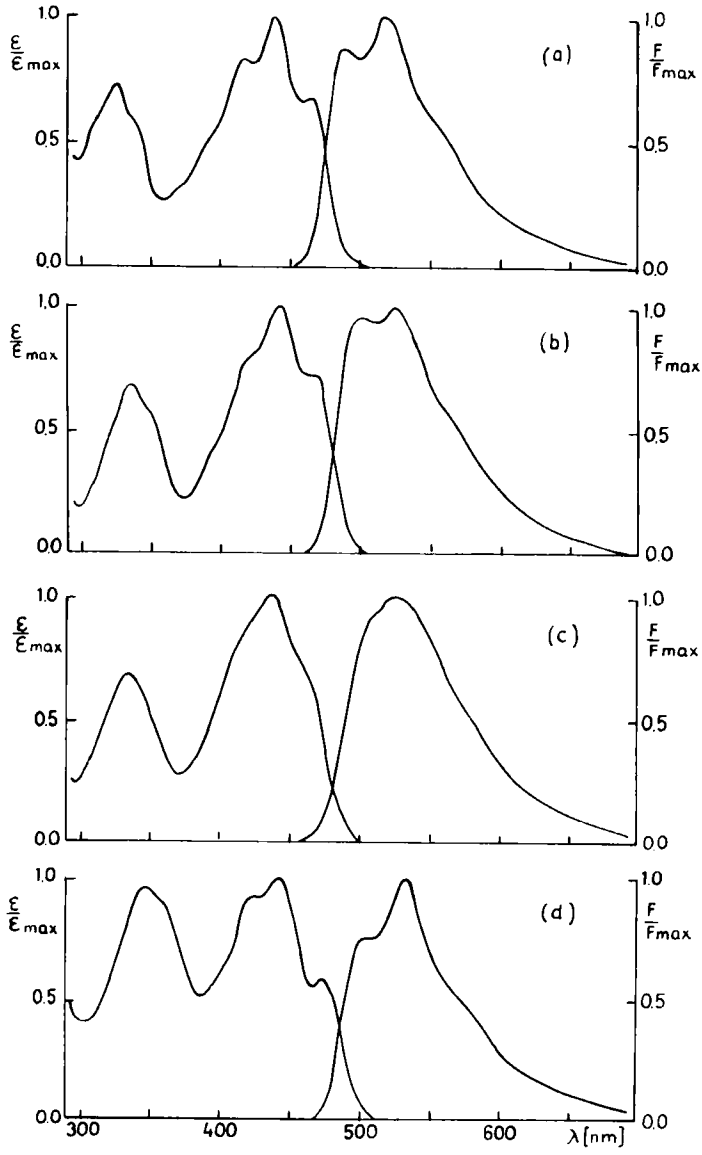


FIG. 2. Absorption and fluorescence spectra of 10-dodecyl-isoalloxazine in: carbon tetrachloride - (a), chloroform - (b), ethanol - (c), 95% water / 5% ethanol - (d).

RESULTS AND DISCUSSION

The results of investigations of electronic absorption spectra (Fig. 2, Tab. 1), fluorescence spectra (Fig. 2, Tab. 2), quantum yields (Tab. 3) and fluorescence decay time (Tab. 3) of amphiflavins in carbon tetrachloride, chloroform and ethanol show that the behaviour of the investigated spectral properties in the function of solvent polarity change is typical for most flavins. In carbon tetrachloride, as in most non-polar solvents, the vibronic structure of absorption and the fluorescence spectra of amphiflavins are very distinct (Fig. 2a). The lifetimes in the excited state and the quantum yield of fluorescence are the greatest. On passing to a slightly less polar solvent, such as chloroform, the structuralization of spectra becomes less distinct (Fig. 2b), and the decay times and quantum yield of fluorescence decrease. Neither carbon tetrachloride nor chloroform form hydrogen bonds with amphiflavins. These solvents perfectly solvate all the hydrophobic regions of the investigated molecules, preventing the so-called stacking interactions between amphiflavin molecules. The observed structuralization of spectra is typical when interactions between solvent and the dissolved molecules are weak.

On passing to ethanol almost complete disappearance of spectra structuralization can be observed (Fig. 2c), as well as a further slight decrease of quantum yield and fluorescence decay times. Ethanol forms hydrogen bonds with amphiflavins. Their formation accelerates the disappearance of the absorption and fluorescence spectra vibronic structure. Experiments with phase-sensitive spectra and fluorescence decay times of amphiflavins have shown that in each of the solvents used only one type of fluorescence centres exists.

TABLE 1.

Absorption maxima (nm) of amphiflavins in various solvents.

Solvent	DIA		OIA		MDIA	
	S2	S1	S2	S1	S2	S1
carbon tetrachloride	325	414 439 464	325	414 439 464	326	417 440 466
chloroform	334	s420 441 s465	334	s420 441 s465	338	s420 443 s467
ethanol	333	436	334	436	332	437
95% water (* 5% ethanol	348	425 443 473	349	425 444 473	351	430 448 480

Concentration of amphiflavins were 10μM, s denotes shoulder, (* OIA and MDIA concentration was 5μM.

TABLE 2.

Fluorescence maxima (nm) of amphiflavins in various solvents.

Solvent	DIA	OIA	MDIA
carbon tetrachloride	495 520	495 520	496 521
chloroform	502 526	502 526	504 528
ethanol	528	528	530
95% water (* 5% ethanol	510 534	510 534	510 535

Concentration of amphiflavins were 10μM, (* OIA and MDIA concentration was 5μM.

TABLE 3.

Decay times (τ) and quantum yields (η) of amphiflavins in organic solvents.

Solvent	DIA		OIA		MDIA	
	τ(ns)	η	τ(ns)	η	τ(ns)	η
carbon tetrachloride	6.7	0.49	6.7	0.49	6.6	0.48
chloroform	6.0	0.39	6.0	0.39	5.9	0.37
ethanol	5.5	0.31	5.4	0.32	5.2	0.30

The error of τ measurements was less than 0.1ns.
The error of η measurements was less than 0.01.

TABLE 4.

Fluorescence anisotropy of amphiflavins in binary solvent.

conc. (μM)	ethanol (% vol.)	λ_{ex} (nm)	DIA	OIA	MDIA
5	40	334	0.03	0.10	0.10
		435	0.06	0.15	0.15
10	40	334	0.11	0.16	0.15
		435	0.14	0.21	0.21
20	40	334	0.16	0.19	0.18
		435	0.21	0.25	0.27
40	40	334	0.18	0.21	0.22
		435	0.23	0.32	0.34
80	40	334	0.19	-	-
		435	0.27	-	-
10	20	334	0.14	0.18	0.17
		435	0.20	0.25	0.26
10	10	334	0.18	0.22	0.23
		435	0.24	0.33	0.35
10	5	334	0.19	-	-
		435	0.26		

The error of anisotropy measurements was less than 0.02.

Surprising results have been obtained for amphiflavins in a binary solvent (water/ethanol) (Fig.3, Tab.4, Tab.5). From the spectral investigation point of view ethanol/water mixtures cannot be considered as only solvents with adjusted polarity, depending on the proportion of the mixture components. Our experiments indicate the presence of some processes leading to a selforganization of amphiflavin molecules in a binary solvent, similarly to the case of lipids in water. Solvation of a long hydrocarbon chain in the N(10) position and of the isoalloxazine ring benzene part of the investigated amphiflavins by water is energetically very inconvenient. Hence, selforganization of flavins into structures in which dye molecules get into a closer contact may occur. This assumption is confirmed by the natural tendency of even polar flavins to form stacking structures in water [19-22]. It is also confirmed by the practical insolubility of the investigated amphiflavins in water. However, results presented by Shinkai [13,14] for 3-methyl-10-dodecylisoalloxazine (MDIA) at a 50μM concentration in water are incomprehensible. Insolubility of similar isoalloxazine derivatives i.e. 7,8,10-trimethyl-3-octadecylisoalloxazine and 3-methyl-10-octadecylisoalloxazine was also observed by Frehland [23]. Absorption spectra in ethanol/water mixtures of low ethanol content presented by Shinkai are in agreement with our results [24,25]. The observed changes in MDIA absorption spectra in a binary solvent are ascribed by Shinkai to the formation of stacking structures of the dimer, trimer, tetramer, etc. type.

Our investigations of absorption spectra and fluorescence spectra (Fig.3), anisotropy (Tab.4), decay times (Tab.5) and fluorescence quantum yield (Tab.5) of amphiflavins in a binary solvent do not negate Shinkai's thesis but show the necessity for its substantial

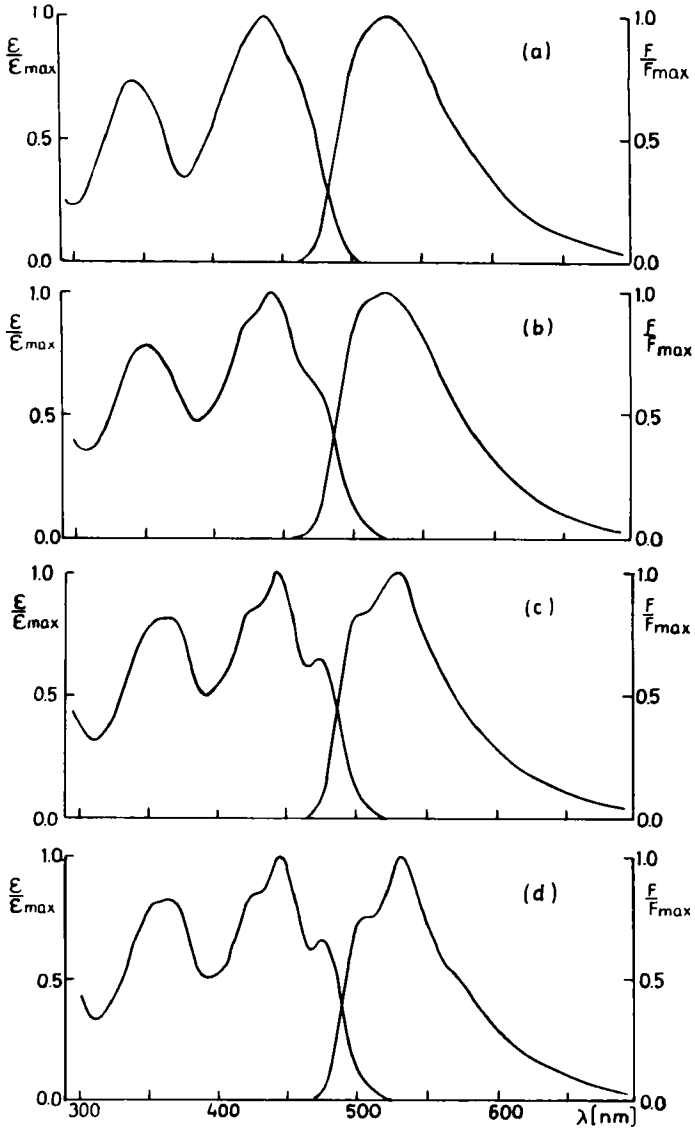


FIG. 3. Absorption and fluorescence spectra of 10-dodecyl-isoalloxazine in 60% water / 40% ethanol. Dye concentrations: 5μM - (a), 10μM - (b), 40μM - (c), 80μM -(d).

TABLE 5.

Average decay times and quantum yields of amphiflavins in binary solvent.

conc. (μM)	ethanol (% vol.)	DIA		OIA		MDIA	
		τ(ns)	η	τ(ns)	η	τ(ns)	η
5	40	4.8	0.21	4.4	0.16	4.2	0.14
10	40	4.5	0.15	3.3	0.12	3.1	0.10
20	40	3.4	0.11	2.6	0.08	2.4	0.07
40	40	2.6	0.08	1.3	0.04	1.3	0.03
80	40	1.8	0.05	-	-	-	-
10	10	2.5	0.08	1.4	0.04	1.3	0.03
10	5	1.5	0.05	-	-	-	-

The error of τ measurements was less than 0.1ns.
The error of η measurements was less than 0.01.

modification. The occurrence of a structuralized fluorescence band of amphiflavins, slightly shifted towards longer wavelengths in relation to non-polar solvents and ethanol, cannot be the result of stacking configurations and are non-fluorescent in most cases. Yet, it can be assumed that observed fluorescence comes from single molecules of amphiflavins found in various fluorescence centres. The results of experiments with small 'effective' concentrations (concentration in ethanol before mixing with water) of amphiflavins in a binary solvent show that one type of these centres is made up by chromophores solvated mostly by ethanol (Fig.3). The increase of effective concentration causes the increase of the number of molecules taking part in selforganizing structures, becoming another kind of fluorescence centre with properties different from the

previous one. It seems that for given ratios of water to ethanol the number of centres solvated mainly by ethanol is constant, and the amphiflavin concentration increase causes the dominance of fluorescence coming from molecules taking part in organized structures. This confirms the observed increase of anisotropy (Tab. 4), the quantum yield decrease (Tab. 5) and the shortening of fluorescence decay times (Tab. 5) along with the increase of amphiflavin concentration in solution. The considerable increase of fluorescence anisotropy along with the increase of amphiflavin 'effective' concentration is caused by the following three effects: (i) - when forming structures amphiflavin molecules are tightly packed and ordered this limiting their thermal movements, (ii) - the increase of concentration quenching of fluorescence diminishes the participation of depolarized fluorescence of molecules secondarily excited as the result of energy transfer, (iii) - the increase of the number of molecules taking part in selforganizing structures.

The selforganization results in the increase of local concentration of amphiflavins which, along with molecule ordering, diminishes (as a concentration quenching result) the lifetime in the excited state and the fluorescence quantum yield. Since selforganization may lead to the formation of dimers and higher ordered stacked associates, non-active absorption of excitation energy by the associated molecules is quite possible.

Investigations of phase-sensitive fluorescence spectra did not bring any decisive results about the existence of various fluorescence centres. However, the results of fluorescence decay time measurements with the pulse method show the multiexponential character of observed decays. It should be assumed that selforganizing structures are not homogenous, and

amphiflavin molecules in such structures may occur in various microenvironments. This brings about the possibility of existence of many various fluorescence centres, some of them being amphiflavins solvated mainly by ethanol, others - amphiflavins involved in organized structures. Distinct spectral properties of amphiflavins involved in various molecular structures show how significant is the influence of flavin interaction with the microenvironment on their spectral properties and microenvironment organization in multicomponent systems.

It should by emphasized that the observed absorption and fluorescence spectra in a binary solvent are very similar to those observed for some flavoproteins. Structuralization of the S1 band of flavins in flavoproteins used to be attributed to the active centre hydrophobicity, but the position of the S2 band, indicating an increased polarity of the microenvironment, seems to contradict this. This may be explained by strong (from the polarity point of view) heterogenity of particular regions of the flavoprotein active centre and of coenzyme stiffening in this centre. The presented results of our experiments clearly show how solvent polarity forces amphiflavin molecules to provide themselves with the energetically most advantageous microenvironments, leading to amphiflavin selforganization. It is a typical case of feedback. In the case of biological systems it may be stated that the structure of the flavoprotein active centre takes into account the influence of coenzyme on the properties of its direct microenvironment.

ACKNOWLEDGEMENTS

The authors thank Dr. P.F. Heelis from the School of Natural Science, North E. Wales, Great Britain for amphiflavins, Dr. A. Balter from the Institute of

Physics, Nicholas Copernicus University, for rendering access to the apparatus for phase-sensitive and pulse measurements, and Dr. K. Bryl for useful discussions. This study was carried out under the RP.II.11 project.

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Date Received: 08/10/89
Date Accepted: 09/12/89