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SPECTROSCOPIC AND DYNAMIC CHARACTERIZATION OF FMN IN REVERSED MICELLES ENTRAPPED WATER POOLS

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Abstract—The encapsulation of FMN in surfactant entrapped water pools resulted into specific interactions of FMN with the polar head groups, the entrapped water molecules and the outer apolar solvent. Two positively charged surfactant/solvent systems were employed: dodecyl ammonium propionate (DAP) in toluene and hexadecyltrimethylammonium bromide (CTAB) in chloroform/*n*-octane (6:5, vol/vol). Also a surfactant with a negatively charged polar head group, sodium *bis* (2-ethylhexyl) sulfosuccinate (AOT) in *n*-octane, was used. In CTAB and especially DAP reversed micellar systems the light absorption spectra revealed the localization of the flavin in a more apolar environment, while in AOT reversed micelles FMN appeared to reside mainly in the core of the water pool. The fluorescence spectra showed unresolved bands, which were blue-shifted in DAP and CTAB reversed micelles as compared to the spectra of aqueous FMN solutions. The fluorescence decay kinetics of FMN in enclosed water droplets is non-exponential. The heterogeneity can be explained assuming incomplete relaxation of partly immobilized water molecules during the lifetime of the excited singlet state. The relatively high anisotropy of the fluorescence of FMN in encapsulated water indicated a higher viscosity than in bulk water. This was confirmed by anisotropy decay measurements of FMN in DAP and AOT entrapped water, for which the rotational correlation times were much longer than for FMN in plain water.

INTRODUCTION

The investigation of surfactant entrapped biomolecules in hydrocarbon solvents is a very active area of research (for recent surveys, see e.g., Luisi and Wolf, 1982 and Fendler, 1982). Recently the entrapment of two redox proteins, cytochrome *c* and cytochrome c_3, was described and it was shown that the native structure could be maintained in the solubilization process (Visser and Fendler, 1982). This prompted us to investigate the stabilization of another class of redox proteins, namely flavoproteins. Because it soon turned out that the spectral properties of entrapped flavins are altered compared to the situation in aqueous solution we undertook a systematic study of the light absorption and fluorescence properties of the prosthetic group FMN‡ (flavin mononucleotide, riboflavin-5′-phosphate) in reversed micelles enclosed water pools.

The spectral properties of the isoalloxazine moiety, the main constituent of the flavin prosthetic group, are known to depend strongly on the polarity and dielectric constant of the solvent (Dudley *et al.*, 1964; Koziol, 1966; Visser and Müller, 1979; Eweg *et al.*, 1979) and also on the presence of hydrogen bonds to specific sites of the isoalloxazine nucleus (Yagi *et al.*, 1980). The variability in optical properties of different flavoproteins also indicated microenvironmental variations of the flavin (Müller *et al.*, 1973). Fluorescence was studied in model flavins in different solvents (Koziol, 1969; Visser and Müller, 1979; Eweg *et al.*, 1979); in flavoproteins (Ghisla *et al.*, 1974; Visser *et al.*, 1974; Veeger *et al.*, 1976; Visser *et al.*, 1980) and in membrane embedded flavins (Heelis *et al.*, 1979; Schmidt, 1979; Schmidt, 1981; Visser, 1982). Several of the more recent results have been reviewed by Müller (1981).

The present work gives a detailed account of the spectroscopic behaviour of reversed micelle entrapped FMN. We have focussed attention to the light absorption and fluorescence spectra and to dynamic properties like anisotropy and fluorescence decay kinetics, from which hydrodynamic information can be inferred. The spectral results could be interpreted by referring to the phenomenological descriptions as given in the above references. The effect of three different surfactants was investigated, two bearing a positive charge [dodecyl ammonium propionate (DAP) and hexadecyltrimethyl ammonium bromide (CTAB)] and one with a negative polar head group [sodium *bis* (2-ethylhexyl)sulfosuccinate (AOT)].

MATERIALS AND METHODS

FMN, purchased from Sigma (St. Louis, MO), was purified on DEAE-Cellulose (Whatman) (Massey and Swoboda, 1963). The AOT was obtained from Serva (Heidelberg) and purified on charcoal according to Calvo-Perez *et al.* (1981). The CTAB was from Serva and

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‡*Abbreviations*: AOT, sodium *bis* (2-ethylhexyl)sulfosuccinate; CTAB, hexadecyl trimethyl ammonium bromide; DAP, dodecyl ammonium propionate; FMN, flavin mononucleotide.

DAP was a gift from Dr. C. Laane, Agricultural University, Wageningen. Reversed micelle entrapped FMN solutions were prepared by dissolving the surfactants in 5 mℓ of organic solvent followed by addition, under mixing, of a predetermined volume of an aqueous FMN stock solution. Quantities were chosen such that the desired w_0, defined as [H$_2$O]/[Surfactant], and a total FMN concentration of around 10 μM were reached. Total surfactant concentrations were as follows: [AOT] = 0.10 M in *n*-octane, [CTAB] = 0.30 M in chloroform/*n*-octane (6:5, vol/vol) and [DAP] = 0.28 M in toluene. The same reversed micelle with the same amount of water but without FMN was used as solvent blank. Light absorption spectra were measured on a Cary-16 spectrophotometer and fluorescence spectra on an Aminco-SPF 500 spectrofluorometer. Absorption and fluorescence spectra were digitized on a PDP-11 graphics system and further processed by the DEC-10 computer Calcomp-1039 plotter of the Computer Center of the Agricultural University. Fluorescence spectra were corrected as previously described (Visser and Müller, 1979). Steady state anisotropy of the fluorescence (excitation 458 nm, emission via Schott KV 500) was determined on a photon counting instrument earlier described (Visser and Santema, 1981). Fluorescence and anisotropy decay were measured on a time resolved single photon counting system with a mode-locked Ar ion laser as excitation source. This system, the tests with single lifetime standards, fluorescence and anisotropy decays and the inherent data analyses have been sufficiently detailed elsewhere (Visser and van Hoek, 1979; Visser and van Hoek, 1981; van Hoek and Visser, 1981; Visser, 1982; Papenhuijzen and Visser, 1983; van Hoek, Vervoort and Visser, 1983). Excitation was at 458 nm and emission was viewed via a Balzers 531 nm interference filter. In all above measurements the ambient temperature was 20°C and the contribution of the solvent blank was subtracted from that arising from entrapped FMN.

RESULTS

Light absorption and fluorescence spectra

Figure 1 shows the absorption spectra of FMN in water surrounded by different surfactants, all scaled to the maximum extinction in the 440–450 nm region. In Figs. 1A–C we have collected the spectra of FMN in the same surfactant but with relatively high and low w_0-values. In Fig. 1D the results in different surfactants with low w_0 are shown in combination with the absorption spectrum of FMN in water. All spectra are different in shape and neither one resembles that of FMN in water. Only in large AOT entrapped water pools with $w_0 = 28.7$ the spectrum is similar to that of aqueous FMN and this is evidence that the flavin is mainly located in the region consisting of bulk water remote from the polar head

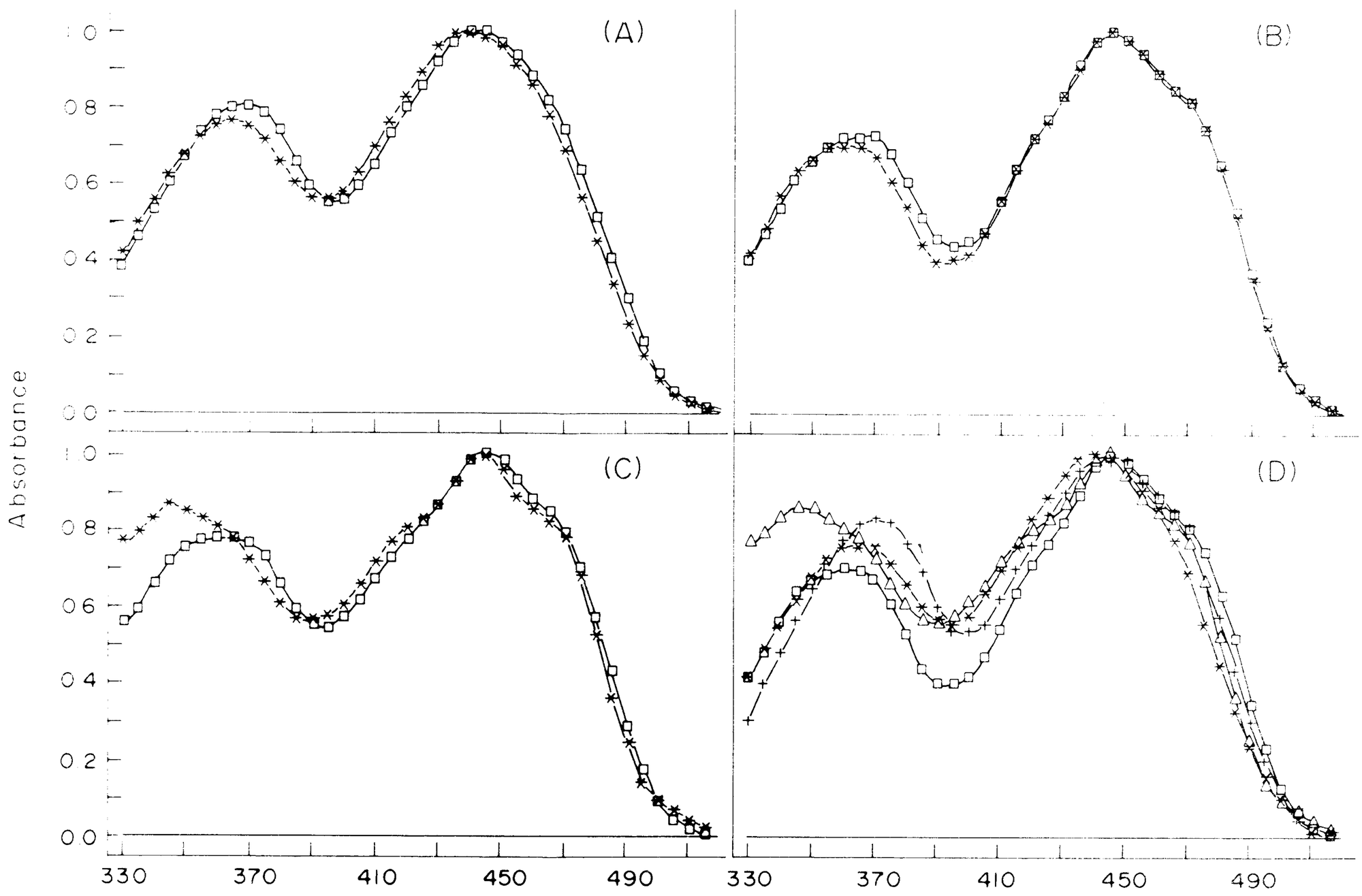

Figure 1. Peak normalized light absorption spectra of FMN in surfactant entrapped water pools. In all examples shown the overall FMN concentration amounted to about 10 μM, the surfactant concentrations in the solvent used were [AOT] = 0.10 M (*n*-octane), [CTAB]=0.30 M (chloroform *n*-octane 6:5, vol vol), [DAP] = 0.28 M (toluene). w_0 is defined as [H$_2$O]/[Surfactant]. (A) FMN in AOT micelles, *** w_0 = 3.8, □□□ w_0 = 28.7. (B) FMN in CTAB micelles, *** w_0 = 1.24, □□□ w_0 = 9.4. (C) FMN in DAP micelles, *** w_0 = 0.25, □□□ w_0 = 4.9. (D) FMN in water and in different micelles, *** AOT w_0 = 3.8, □□□ CTAB w_0 = 1.24, △△△ DAP w_0 = 0.25, +++ FMN in water.

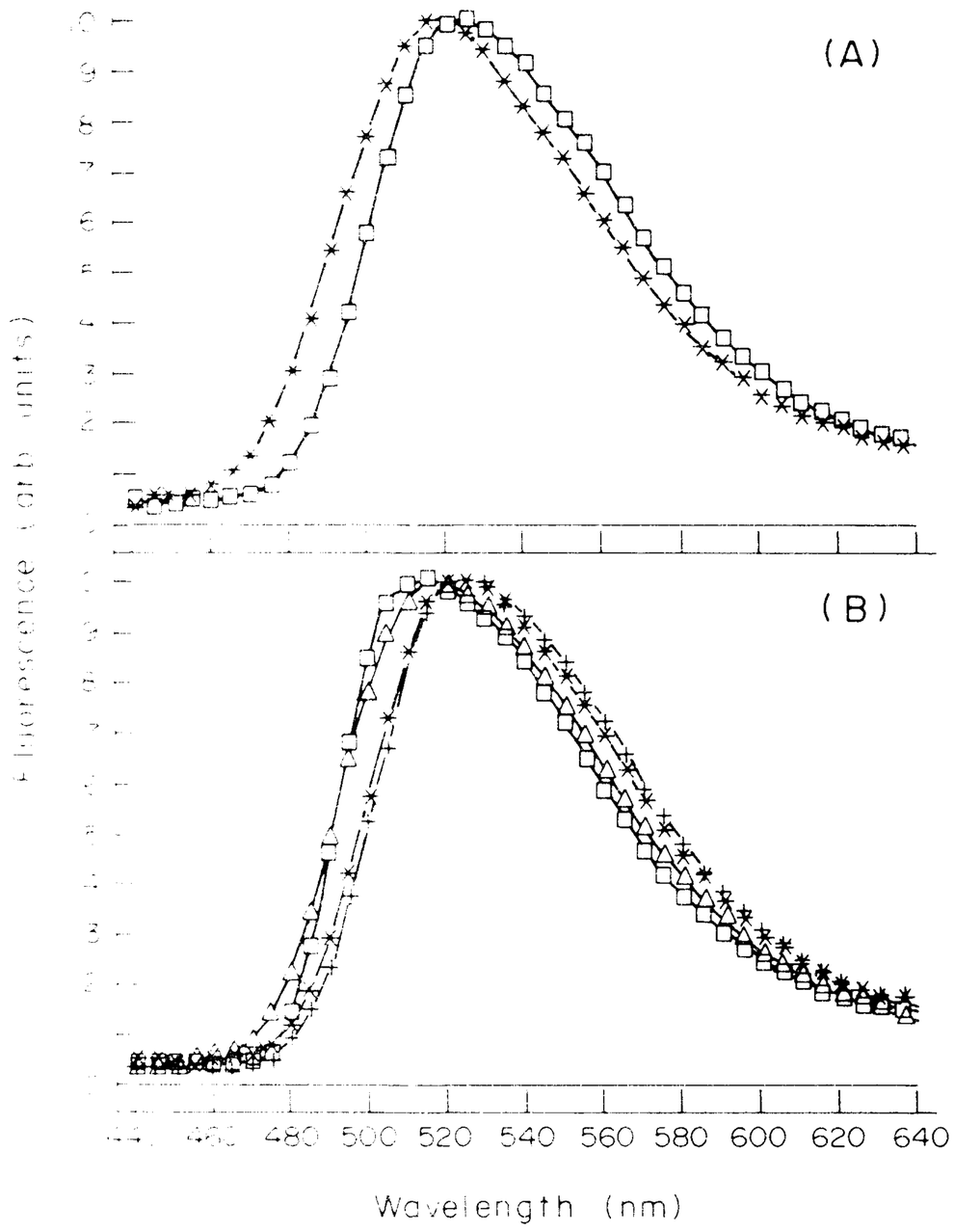

Figure 2. Peak normalized fluorescence spectra of FMN in surfactant entrapped water pools. (A) FMN in AOT, *** w_0 = 3.8, DAP w_0 = 28.7. (B) FMN in water and in different micelles. *** AOT w_0 = 28.7, □□□ w_0 = 4.9, CTAB w_0 = 9.4, +++ FMN in water. Further details as in the legend of Fig. 1.

groups. At low $[H_2O]/[AOT]$ both absorption bands are slightly blue-shifted, but they remain structureless. It is known that the size of the water droplets decreases with lower w_0 (Fendler, 1982) and in that case it can be very well imagined that the microenvironment of FMN consists of water, which is immobilized by virtue of binding to the sulfosuccinate anions. The absorption spectra of FMN in DAP or in CTAB entrapped water can easily be distinguished from that of FMN in AOT inverted micelles. The lowest energy absorption shows a distinct vibronic structure characteristic for flavin in an apolar environment. The second electronic transition is much more sensitive towards solvent polarity changes than the first electronic transition at lower energy. The second absorption band for the DAP system shows the most marked blue-shift (to 345 nm) at low water content.

The fluorescence spectra are characterized by broad structureless bands. The fluorescence spectra of FMN in cationic inverted micelles did not change upon w_0-variation (not shown). Only with AOT a progressive redshift of flavin emission is seen after increasing w_0 (Fig. 2A). In Fig. 2B spectra of FMN in different surfactant solutions (highest w_0) are portrayed together with that of FMN in aqueous solution. There is only overlap between the spectra of FMN in water and of FMN in large water pools encapsulated by AOT. In the water pools surrounded by the cationic surfactants there is a significant blue-shift of about 10 nm.

The quantum yield varies with the nature of the surfactant and counterion. The bromide counterion in CTAB quenches the flavin fluorescence considerably (*vide infra*). In Table 1 the quantum efficiencies relative to that of FMN in aqueous solution are compiled.

Lifetimes and anisotropy of fluorescence

In water, FMN is a very suitable single lifetime standard (τ = 4.7 ns, Wahl *et al.*, 1974). We have used FMN in water as a test sample for our decay fluorometer and the results are shown in Fig. 3A. The fluorescence response to the exciting laser pulse is extremely well fitted to a single exponential function

Table 1. Fluorescence characteristics of FMN in surfactant enclosed water pools

Sample	w_0	Relative quantum efficiencies	Fluorescence lifetimes				
			α_1	τ_1 (ns)	α_2	τ_2 (ns)	$\langle\tau\rangle^*$ (ns)
FMN H$_2$O	—	1.00		4.70			4.70
FMN AOT	3.8	0.98	0.76	4.65	0.24	0.79	4.45
FMN AOT	28.7	1.05	0.57	4.75	0.43	0.16	4.64
FMN H$_2$O 0.5 *M* KBr	—	0.072	0.78	0.26	0.22	0.58	0.38
FMN CTAB	1.2	0.090	0.29	1.09	0.71	0.23	0.78
FMN CTAB	9.4	0.083	0.33	0.71	0.67	0.24	0.52
FMN DAP	0.25	0.51	0.60	4.42	0.40	1.03	3.96
FMN DAP	2.1	0.52	0.64	3.70	0.36	1.63	3.29
FMN DAP	4.9	0.52	0.73	3.73	0.27	0.94	3.49

*Average lifetime defined as $\langle\tau\rangle = \sum_{i=1}^{2} \alpha_i\tau_i^2 / \sum_{i=1}^{2} \alpha_i\tau_i$.

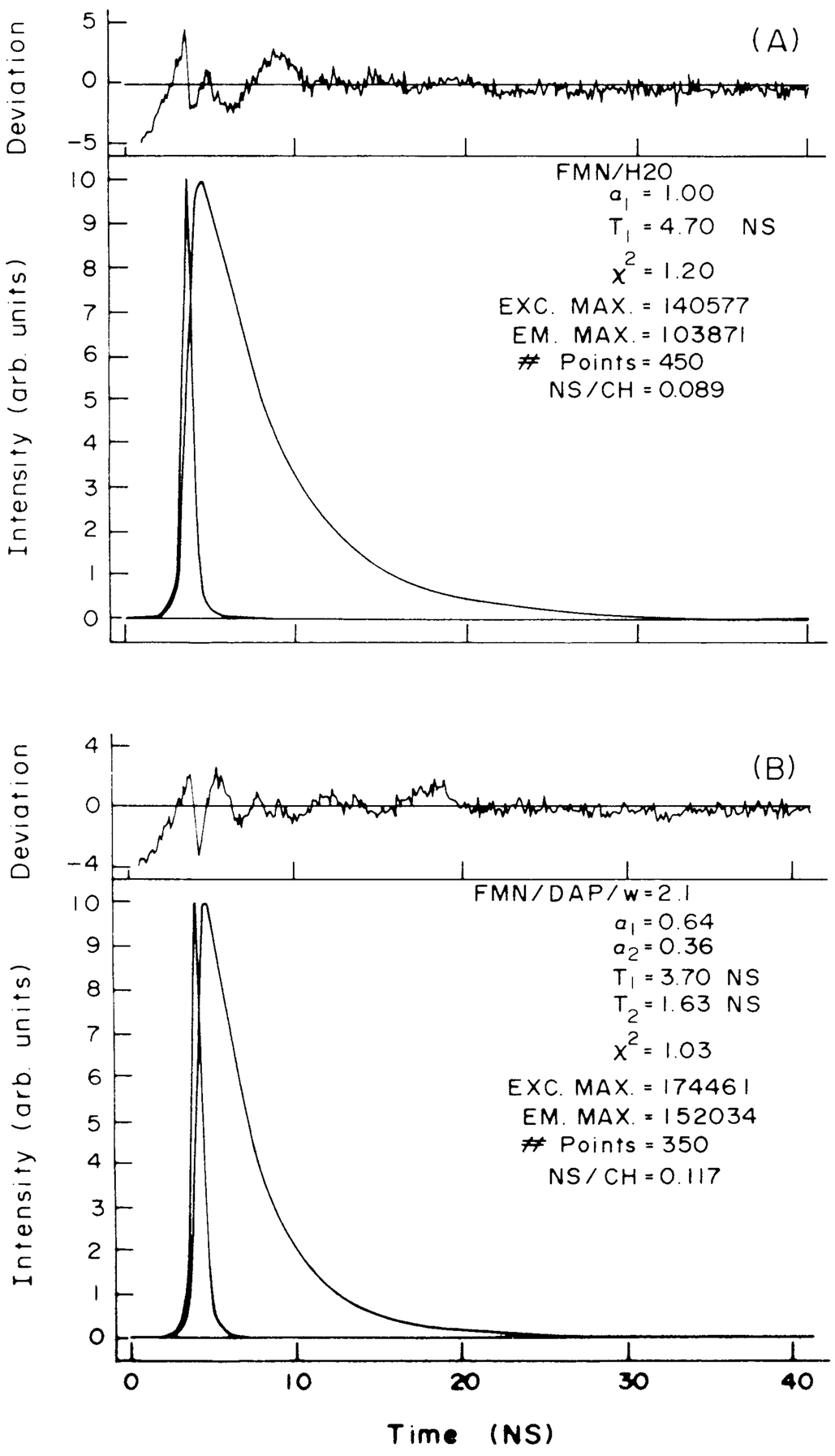

Figure 3. (A) Fluorescence decay of FMN in water (pH 7.0). Shown are the laser pulse profile, the experimental fluorescence [$F(t) = F_{\parallel}(t) + 2F_{\perp}(t)$] and fitted fluorescence (F_c) composed of the convolution product of the laser pulse with a single-exponential decay function. The deviation function in each channel i (top panel) is given by $[F_c(i) - F(i)] \times W(i)$ where $W(i) = 1/F(i)$. The values of the parameters (α_1, τ_1) of the exponential decay model are listed. Additional information is presented such as number of counts in the maxima of laser pulse (EXCMAX) and experimental fluorescence (EMMAX), number of data channels used and time equivalence per channel. The χ^2-value has been calculated after normalization of EXCMAX and EMMAX to 10000. (B) Fluorescence decay of FMN in DAP reversed micelle entrapped water (w_0 = 2.1) and fit to a double exponential decay model.

with a time constant of 4.7 ns. The figure of merit in the fitting procedure is the minimum value of χ^2, which is actually obtained after a single exponential analysis of the fluorescence-time profile of this reference compound. The fluorescence decay of FMN in surfactant enclosed water pools proved to be non-exponential. Analysis into two lifetime components always yielded much lower χ^2 and plots of weighted residuals, which are fluctuating around the baseline. One example is shown in Fig. 3B. The fluorescence lifetimes and relative amplitudes are listed in Table 1. It is noticeable that the long lifetime component in AOT enclosed water is equal to the single lifetime of FMN in AOT inverted micelles. The lifetimes in DAP reversed micelles (Fig. 3B) were shorter than the FMN reference, but the shortest lifetimes were found in CTAB micelles (Table 1). The latter quenching is due to collisions with bromide anions and the effect could be demonstrated in aqueous FMN upon addition of 0.5 *M* KBr leading to an average fluorescence lifetime of 0.38 ns (Table 1).

The enhanced viscosity of the entrapped water is manifested by the presence of distinct non-zero anisotropy ($<r>$) of FMN fluorescence in all micelles studied (Table 2). The high anisotropy of CTAB reversed micelles originates from the short average fluorescence lifetime caused by quenching by bromide. In AOT reversed micelles the anisotropy

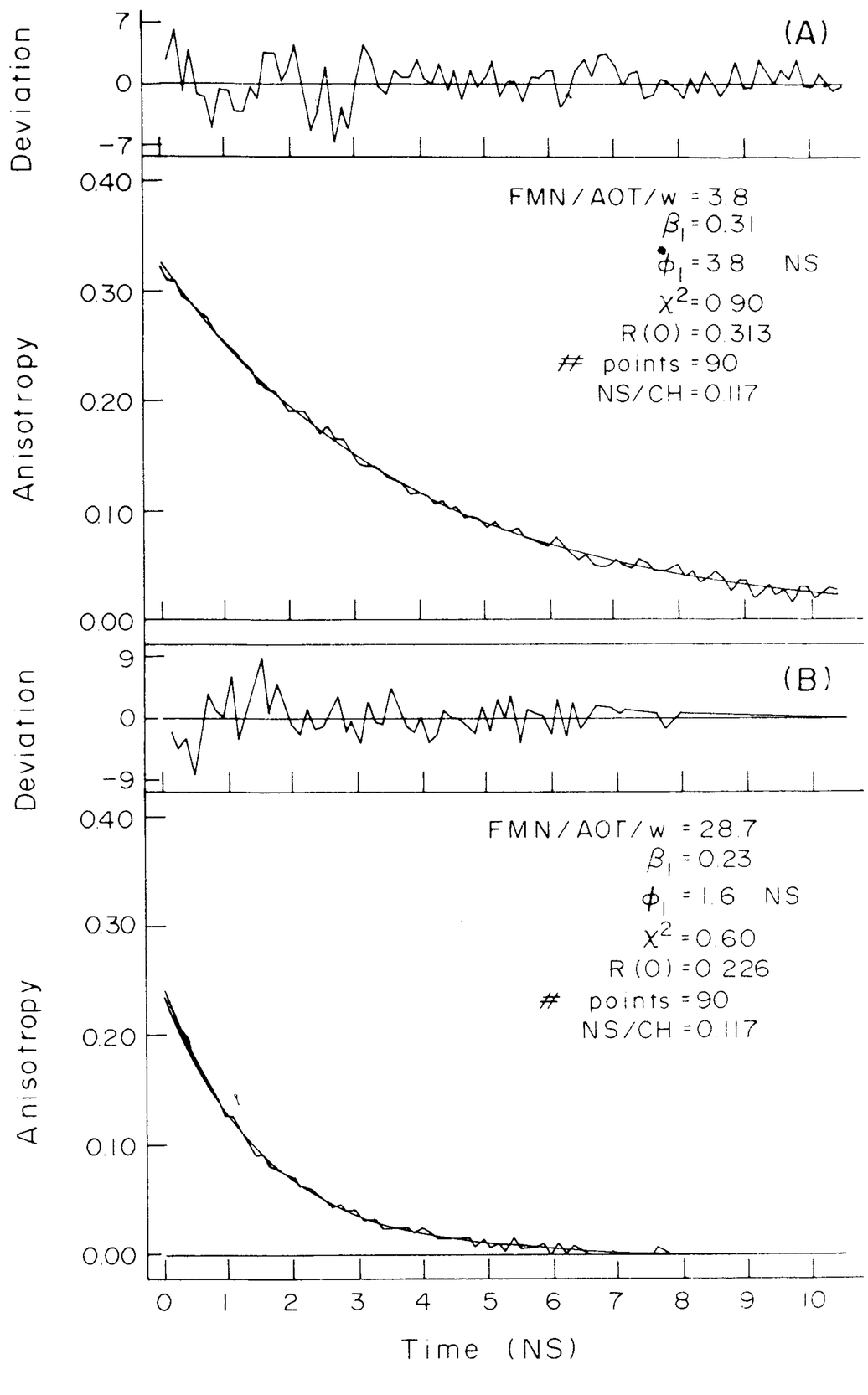

Figure 4. A. Decay of fluorescence anisotropy of FMN in AOT reversed micelle entrapped water. Shown are the experimental anisotopy, $r(t) = [F_{\parallel}(t) - F_{\perp}(t)]/[F_{\parallel}(t) + 2F_{\perp}(t)]$, and the fit to an exponential function $r_c(t) = \beta_1 \exp(-t/\phi_1)$. The parameters β_1 [$\equiv r(0)$] and ϕ_1 are given together with other information as detailed in the legend of Fig. 3. The deviation function in each channel i (top panel) is defined by $[r_c(i) - r(i)] \times W(i)$, where the weighting factor $W(i)$ is given by $W(i) = 1/V(i)$, $V(i)$ is the variance of $r(i)$. χ^2 has been multiplied with 100000. The analysis started at EXCMAX (see legend Fig. 3) and the data were not deconvoluted from the laser pulse. (A) w_0 = 3.8. (B) w_0 = 28.7.

Table 2. Anisotropy characteristics of FMN in surfactant enclosed water pools

Sample	w_0	$<r>$	ϕ^* (ns)	R_h† (Å)	ϕ_m‡ (ns)	ϕ_i§ (ns)
FMN/AOT	3.8	0.175	3.8(4.0)	25	8.8	6.7
FMN/AOT	28.7	0.075	1.6(1.2)	55	93.3	1.6
FMN/CTAB	1.2	0.215	(1.1)	—	—	—
FMN/CTAB	9.4	0.206	(0.7)	—	—	—
FMN/DAP	0.25	0.059	1.4(1.3)	—	—	—
FMN/DAP	2.1	0.099	1.8(1.2)	21	5.7	2.6
FMN/DAP	4.9	0.100	2.1(1.3)	26	10.7	2.5

*Experimental correlation times, values between brackets are obtained from the Perrin formula: $r_0/<r> - 1 = <\tau>/\phi$. †Hydrodynamic radii of AOT (Zulauf and Eicke, 1979) and of DAP (Fendler, 1982) inverted micelles. ‡From $\phi_m = 4\pi R_h^3\eta/(3kT)$, η (toluene) = 0.59 cp, η (*n*-octane) = 0.54 cp at T = 293 K (Handbook of Chemistry and Physics, 56th ed.). §From $1/\phi_i = 1/\phi - 1/\phi_m$.

depends on w_0, which is lower in large water pools. This tendency is not observed in water droplets surrounded by cationic surfactants (Table 2). The conclusions derived from the average (time-independent) anisotropy were confirmed by the results of anisotropy decay measurements. From deconvolved anisotropy decay of FMN in water a rotational correlation time (ϕ) was determined as 0.2 ns (A.J.W.G. Visser, unpublished). The correlation times of FMN in DAP reversed micelles were much longer and they tend to lengthen with increasing pool size (Table 2). In AOT reversed micelles the correlation times became shorter with larger water pools (Fig. 4, Table 2). In case of CTAB the correlation times (Table 2) were obtained from the Perrin formula: $r_0/<r> = 1 + <\tau>/\phi$, where $<\tau>$ is the average fluorescence lifetime (Table 1) and r_0 is the limiting anisotropy (0.38 for flavins as determined by Visser *et al.*, 1983). It should be noted that in contrast to the fluorescence all anisotropies decayed exponentially.

DISCUSSION

The spectral results reveal several characteristic features of FMN in the surfactant enclosed water pools. The second electronic transition is very sensitive for the amount of hydrogen bonding as indicated by the marked bathochromic shift (Müller *et al.*, 1973; Yagi *et al.*, 1980; Eweg *et al.*, 1982). Therefore we conclude that FMN is somewhat shielded from water in DAP and CTAB entrapped water pools and exposed to water in AOT reversed micelles. It is also possible that solvent molecules like toluene and chloroform might penetrate into the interface region between the water boundary and the polar head group of the surfactant as to bring the solvent more in contact with flavin. Electrostatic interactions also play a role, since the negative phosphate group of FMN is repelled from the negatively charged sulfonate in AOT, whereas it will be attracted by the positive quaternary ammonium ions of DAP and CTAB surfactants. The amount of shielding or exposure as manifested by spectral changes depends on the size of the water pools controlled by increasing the amount of water at constant surfactant concentration. In small sized droplets the water molecules are used up in binding to the polar head groups via ion–dipole and dipole–dipole interactions and in counterion solvation. The nature of this immobilized water is different from that in larger-sized water clusters. Such a conclusion was reached for the AOT–H_2O–heptane system by Wong *et al.* (1975) by using fluorescent polarity probes and for the DAP–H_2O–benzene system by Lim and Fendler (1978) via spin labels.

The fluorescence spectral distribution of FMN does not show the vibronic structure as apparent in flavin models in apolar solvents (Eweg *et al.*, 1979) or in some flavoproteins (Visser *et al.*, 1974; Veeger *et al.*, 1976). This indicates that water molecules are able to diffuse to the flavin during its excited state lifetime in CTAB and DAP inverted micelles. Moreover in water clusters surrounded by CTAB the strong quenching of fluorescence is induced by collisional contact of bromide ions suggesting that the flavin is not heavily buried. The non-exponential fluorescence decay must be ascribed to incomplete solvent relaxation processes. With the exception of FMN in water heterogeneous fluorescence decay is a common phenomenon for flavins bound in flavoproteins (Grande *et al.*, 1980; Visser *et al.*, 1980), embedded in membranes (Visser, 1982) or dissolved in a viscous solvent like glycerol (Veeger *et al.*, 1980). The exponential decay of the emission anisotropy proves that the flavin is not partitioned between different phases, for instance located in the core of the water cluster or near the interface region. If that is the case different mobilities and correlation times would be anticipated.

The aggregation state of especially AOT inverted micelles in apolar solvents has been studied with

various techniques like photon correlation spectroscopy (Day *et al.*, 1979; Zulauf and Eicke, 1979) and fluorescence polarization of an embedded probe (Keh and Valeur, 1981). Both types of experiments yielded the Stokes or hydrodynamic radius of reversed micelles. From fluorescence polarization data Keh and Valeur concluded that two coupled rotational diffusion coefficients exist, namely one of the spherical micelle itself (D_m) and an internal diffusion coefficient (D_i), describing isotropic, internal motions of the fluorophore. The time dependence of the antisotropy is then described by $r(t) = \beta_1 \exp(-t/\phi_i) - \beta_2 \exp(-t/\phi_m)$, where $\phi_i = 1/6D_i$ and $\phi_m = 1/6D_m$ (Keh and Valeur, 1981). Our anisotropy decay results are consistent with the antisotropy following a single exponential decay function to be described by $r(t) = \beta_1 \exp[-t(1/\phi_i + 1/\phi_m)]$, thus by independent, uncoupled rotations at the range of w_0-values used. The micellar correlation time ϕ_m can be calculated from the Stokes–Einstein formula using the hydrodynamic radii as determined by Zulauf and Eicke (1979) for AOT-droplets and summarized by Fendler (1982) for the DAP system. From the observed 'effective' correlation time the internal correlation time ϕ_i can be derived. We have incorporated these results in Table 2. From an appraisal of the data in Table 2 it can be seen that ϕ_i becomes distinctly shorter at larger AOT surrounded water droplets, while ϕ_i for the DAP micelles is more or less constant.

In conclusion, the spectral properties of FMN in different reversed micellar systems can be qualitatively interpreted. The spectral results are complemented by (hydro)dynamic information. The specific interactions with charged molecules and with solvent molecules of different polarities as assessed by this study are at least in part responsible for the large variety of 'environmental' features observed in different flavoproteins.

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