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QUARTERLY

Fluorescence decay time distribution analysis of cyclic enkephalin analogues; Influence of solvent and Leu configuration in position 5 on conformation★✪

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Lifetime distribution analysis were performed to study the influence of Leu configuration in position 5 on changes of the peptide chain of cyclic analogues of enkephalins containing a fluorescence donor and acceptor in different solvents. The configuration change of Leu5 in all the analogues of enkephalins studied which contain donor-acceptor pairs has no apparent influence on Trp lifetime distributions. In contrast, there is a significant solvent effect on the shape of lifetime distribution.

The opioid system plays a crucial role in analgesic action. The main elements of this system are endogenous peptides (enkephalins, endomorphins, β-endorphin, dynorphins) which are the natural ligands for opioid receptors (μ, δ, κ). The μ-opioid receptors have a great significance in analgesic action. Structure-biological activity analysis of opioid peptide analogues has shown that rigidifying the peptide chain (which is accomplished by cyclization) results in an increase of their affinity for opioid receptors, probably by stabilizing the bioactive conformation(s) [1, 2]. Mu-

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Abbreviations: MeOH, methanol; MeCN, acetonitrile; Me_2SO, dimethyl sulfoxide; Dns, 5-dimethylaminonaphtalene-1-sulfonyl (dansyl); Phe(p-NO_2), *p*-nitrophenylalanine; Dab, α,γ-diaminobutyric acid; Dns-[L-EN], Dns-*cyclo*[D-Dab^2-Gly^3-Trp^4-Leu^5]; Dns-[D-EN], Dns-*cyclo*[D-Dab^2-Gly^3-Trp^4-D-Leu^5]; F(NO_2)-[L-EN], Phe(p-NO_2)^1-*cyclo*[D-Dab^2-Gly^3-Trp^4-Leu^5]; F(NO_2)-[D-EN], Phe(p-NO_2)^1-*cyclo*[D-Dab^2-Gly^3-Trp^4-D-Leu^5]; Fmoc, fluorene-9-yl-methoxycarbonyl; HBTU, 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate; ^{1}H NMR COSY, hydrogen nuclear magnetic resonance correlation spectroscopy; RP-HPLC, reversed phase high performance liquid chromatography; FAB-MS, fast atom bombardment mass spectrometry.

tual orientation and the distance between the aromatic side chains, as well as their mobility are also important [3–8]. In conformational studies of the opioid peptides, NMR and fluorescence spectroscopy methods and theoretical calculations are widely used. Fluorescence lifetime distribution analysis is one of the fluorescence methods used in the determination of the conformational space of the fluorophores [9–12]. This method gives information on conformational differences of the main peptide chain, (number of modes of distributions) and conformational freedom of the chromophores which characterize a particular family of conformations (width of the distributions). Although this method gives only a qualitative description of the conformational space, it enables a comparison with the data obtained with other methods, (for example, Förster resonance energy transfer (FRET) [13, 14] or molecular dynamics [3, 5, 15]). The FRET methods give qualitative information about distance and distance distribution between chromophores, but it is restricted by the assumption of a given functional form of the distribution (Gaussian or Lorentzian). This limitation is not imposed in the case of the lifetime distribution methods based on the maximum entropy approach.

We applied the lifetime distribution analysis of the tryptophan fluorophore to study the influence of the solvent (H_2O, MeOH, MeCN and Me_2SO) and the configuration of leucine in position 5 on the conformation of the following cyclic analogues

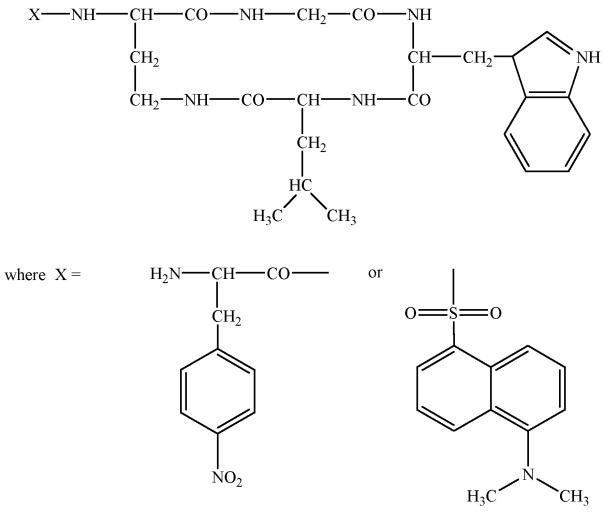

Figure 1. Structure of the cyclic enkephalin analogues considered in this study.

of enkephalins containing a fluorescence energy donor (Trp) and acceptor (Dns or Phe(p-NO_2)): Dns-[L-EN], F(NO_2)-[L-EN], Dns-[D-EN], F(NO_2)-[D-EN].

The structure of the cyclic enkephalin analogues studied is presented in Fig. 1.

The selection of two enkephalin analogues differing in Leu^5 configuration for this studies was caused by their different biological activity [5]. In our studies we used the solvents possessing diverse polarity and ability to hydrogen bond formation to mimic the possible microenvironment of the receptor pocket.

THEORY

The theory of lifetime distribution calculation is described in many papers [9–11, 16, 17]; therefore we present only a brief description here. Fluorescence intensity decays are usually described as sums of individual exponentials. The intensity decay following an infinitesimal pulse excitation (δ-function) is described by:

$$I(t)=\sum_i \alpha_i e^{-t/\tau_i} \qquad (1)$$

where τ_i, i = 1,2,3, are the individual decay times and α_i, i = 1,2,3, the associated pre-exponential factors normalized to unity

$$(\sum_i \alpha_i = 1.0).$$

Now let us consider an alternative model in which the α_i, i = 1,2,..., values are not discrete amplitudes corresponding to decay times τ_i, i = 1,2,..., but are described by a continuous distribution $\alpha(\tau)$. The intensity decay then contains components of each lifetime (τ) with amplitude $\alpha(\tau)$. The total fluorescence intensity decay I(t) is expressed by Eqn. (2):

$$I(t) = \int_{\tau=0}^{\infty} \alpha(\tau) e^{-t/\tau} d\tau \qquad (2)$$

where $\int \alpha(\tau) d\tau = 1.0$. At present no theoretical model exists to predict the $\alpha(\tau)$ distributions and the available experimental data do not suggest any general functional form. Hence arbitrarily selected Gaussian or Lorentzian distributions are

used [10, 16–18]. An alternative approach is not to assume any functional form of $\alpha(\tau)$, but to determine it from the experimental data. This approach is superior in that it makes no assumptions about the shape of the distribution. Two general algorithms: the maximum entropy method [11, 12, 19–22] and the exponential series methods are used for such calculations [23, 24]. For time-correlated single photon counting the measured fluorescence decay $F(t)$ is a convolution of the instrument response function $R(t)$ and the intensity decay function $I(t)$:

$$F(t) = \int_0^t R(s+\delta) I(t-s) ds \qquad (3)$$

where δ is the time shift parameter which takes care of the experimental or computational artifacts which cause an artificial time-shift of the calculated function with respect to experimental data. $I(t)$ is the theoretical intensity decay function, a continuous distribution of lifetimes, as described in equation (2). $\alpha(\tau)$ is the distribution function which is to be determined. The quality of the fit to experimental data is checked by the χ^2 statistics:

$$\chi^2 = (1/M) \sum_{i=1}^{N} \{F_e(t_i) - F_e(t_i)\}^2 / \sigma_i^2 \approx 1.0 \qquad (4)$$

where $F_c(t_i)$ is the calculated fluorescence decay at time t_i, $F_e(t_i)$ is the measured decay at time t_i, σ_i is the standard deviation for the i-th data point and M is the number of the degrees of freedom. Usually the good criterion of $\chi^2 \approx 1.0$ could be obtained for many different distributions of $\alpha(\tau)$. The optimum is the one which fits data adequately ($\chi^2 \approx 1.0$) and maximizes the value of the Shanon-Jaynes entropy function S, as defined below:

$$S = -\sum p_i \log p_i \qquad (5)$$

where $p_i = \alpha_i / \Sigma\alpha_i$. If there is a priori knowledge about the distribution (m_i), equation (5) is modified as follows:

$$S = -\sum p_i \log(p_i / m_i) \qquad (6)$$

If the χ^2 criterion is satisfied for many distributions, then the maximum entropy criterion selects that distribution which contains the minimum number of peaks of maximal width in the distribution.

MATERIALS AND METHODS

Synthesis. Linear precursors of peptides (Dns-[L-EN], Dns-[D-EN], F(NO_2)-[L-EN], F(NO_2)-[D-EN]) were synthesized by solid-phase methodology using the Fmoc chemistry [25]. The cyclization was performed using HBTU [26]. The peptides were purified by means of the preparative RP-HPLC. The homogeneity and molecular constitution of the compounds were assessed by analytical RP-HPLC and FAB-MS, and ^{1}H-NMR COSY spectroscopy.

Time-resolved fluorescence measurements. Fluorescence decay times were measured using a time correlated single-photon counting apparatus at the Laboratory of Ultrafast Laser Spectroscopy, Adam Mickiewicz University (Poznań, Poland). The excitation source (λ_{ex} = 280 nm) was a pico/femtosecond laser system (Ti:Saphire "Tsunami" laser pumped with an argon ion laser "BeamLok" 2060) [27]. The emission was detected with a magic angle polarizer at an emission wavelength of λ_{em} = 340 nm. A Ludox solution was used to collect the instrument response. All measurements were performed at 20°C in water, methanol, acetonitrile and Me_2SO. For lifetime distribution calculation the software provided by Edinburgh Analytical Instruments was used.

RESULTS AND DISCUSSION

If the donor and the acceptor remain at a fixed distance the decay of the donor is accelerated by energy transfer, without changing its mono-exponential time dependence. However, if there is a range of donor-acceptor distances, then a range of transfer rates, and hence a distribution of decay times should be observed. If two unique distance distributions are present one should expect a bimodal time distribution. To check how the solvent and different Leu configuration in position 5 influence the distance between the donor in posi-

tion 4 (Trp) and the acceptor (Dns or Phe(NO_2)) in position 1 in the cyclic analogues of enkephalin considered in this study we measured the fluorescence decay times of the donor in the presence of the acceptor in four different solvents (H_2O, MeOH, MeCN, Me_2SO). Fluorescence lifetime distribution parameters for cyclic enkephalin analogues containing the energy donor (Trp) and the acceptors (Dns or Phe(NO_2)) calculated on the basis of the measured fluorescence intensity decay are presented in Table 1 and in a graphical form in Figs. 2 and 3. The presence of the energy acceptor results in a significant decrease of the fluorescence lifetime of tryptophan compared to the isolated donor [28].

The configuration of Leu^5 in all the analogues of enkephalins studied which contain donor-acceptor pairs has no apparent influence on the Trp lifetime distributions. In contrast, there is a significant solvent effect on the shape of the lifetime distribution. In less polar solvents (MeOH, MeCN) the distributions are asymmetric, uni- or bimodal; in bimodal distributions the long-lifetime components have small amplitudes (0.3–2%). The width of the distributions is higher in the dansyl containing enkephalin analogues. In more polar solvents the number of distribution components increases. In Dns-[L or D-EN] they are narrow and well separated. The short-lifetime components have a higher amplitude than the long-lifetime ones. The conformational space of the peptides studied in MeCN and MeOH can be described in terms of a single conformational family, in which the side chains of the aromatic amino

Table 1. Lifetimes distribution parameters of Trp fluorescence (average fluorescence lifetime decays (τ) and amplitudes (f) of particular components) of cyclic enkephalin analogues in different solvents

Analogue	Lifetime [ns]	Amplitude	Lifetime [ns]	Amplitude	Lifetime [ns]	Amplitude	χ^2_R
	τ_1	f_1	τ_2	f_2	τ_3	f_3	
				H_2O			
Dns-[L-EN]	0.07	0.5903	0.25	0.3847	1.17	0.0250	1.08
Dns-[D-EN]	0.10	0.6045	0.24	0.3737	1.03	0.0217	1.04
F(NO_2)-[L-EN]	0.16	0.2118	1.15	0.7782	4.66	0.0100	1.17
F(NO_2)-[D-EN]	0.50	0.9136	1.31	0.0864			1.06
				MeOH			
Dns-[L-EN]	0.17	0.9811	1.94	0.0189			1.02
Dns-[D-EN]	0.15	0.9941	1.73	0.0059			1.12
F(NO_2)-[L-EN]	0.71	1.00					1.09
F(NO_2)-[D-EN]	0.67	1.00					1.18
				MeCN			
Dns-[L-EN]	0.14	0.9893	1.36	0.0078	5.83	0.0029	1.07
Dns-[D-EN]	0.10	0.9972	1.67	0.0028			1.07
F(NO_2)-[L-EN]	0.61	1.00					1.12
F(NO_2)-[D-EN]	0.55	1.00					1.11
				Me_2SO			
Dns-[L-EN]	0.22	0.9559	1.00	0.0315	5.58	0.0125	1.15
Dns-[D-EN]	0.22	0.8680	0.66	0.1241	5.88	0.0079	1.07
F(NO_2)-[L-EN]	0.62	0.5561	1.19	0.4327	4.88	0.0101	1.29
F(NO_2)-[D-EN]	0.56	0.3073	1.31	0.6667	3.08	0.0260	1.17

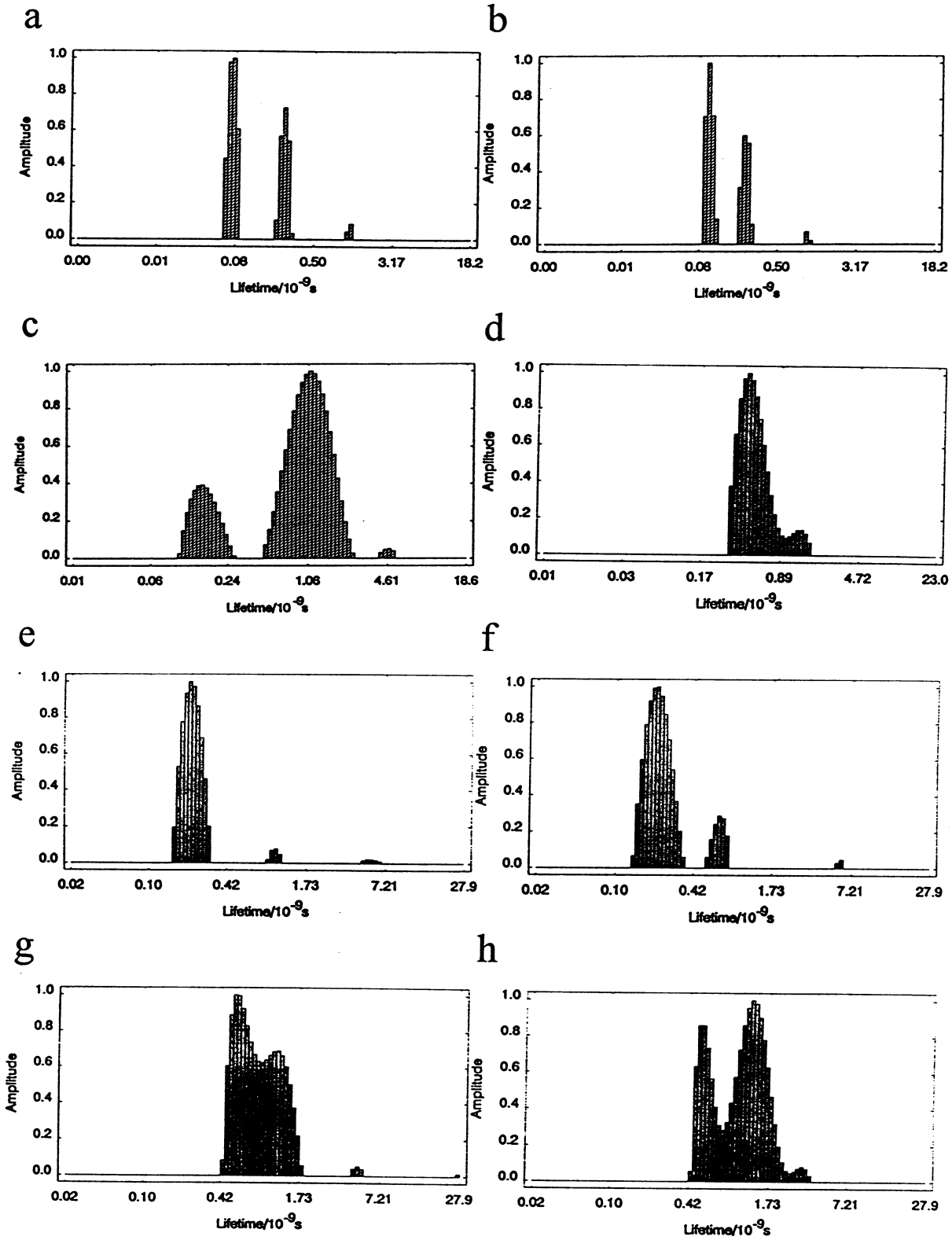

Figure 2. Lifetime distributions of the Trp fluorescence of Dns-[L-EN] (a), Dns-[D-EN] (b), F(NO_2)-[L-EN] (c), F(NO_2)-[D-EN] (d) in water and Dns-[L-EN] (e), Dns-[D-EN] (f), F(NO_2)-[L-EN] (g), F(NO_2)-[D-EN] (h) in Me_2SO.

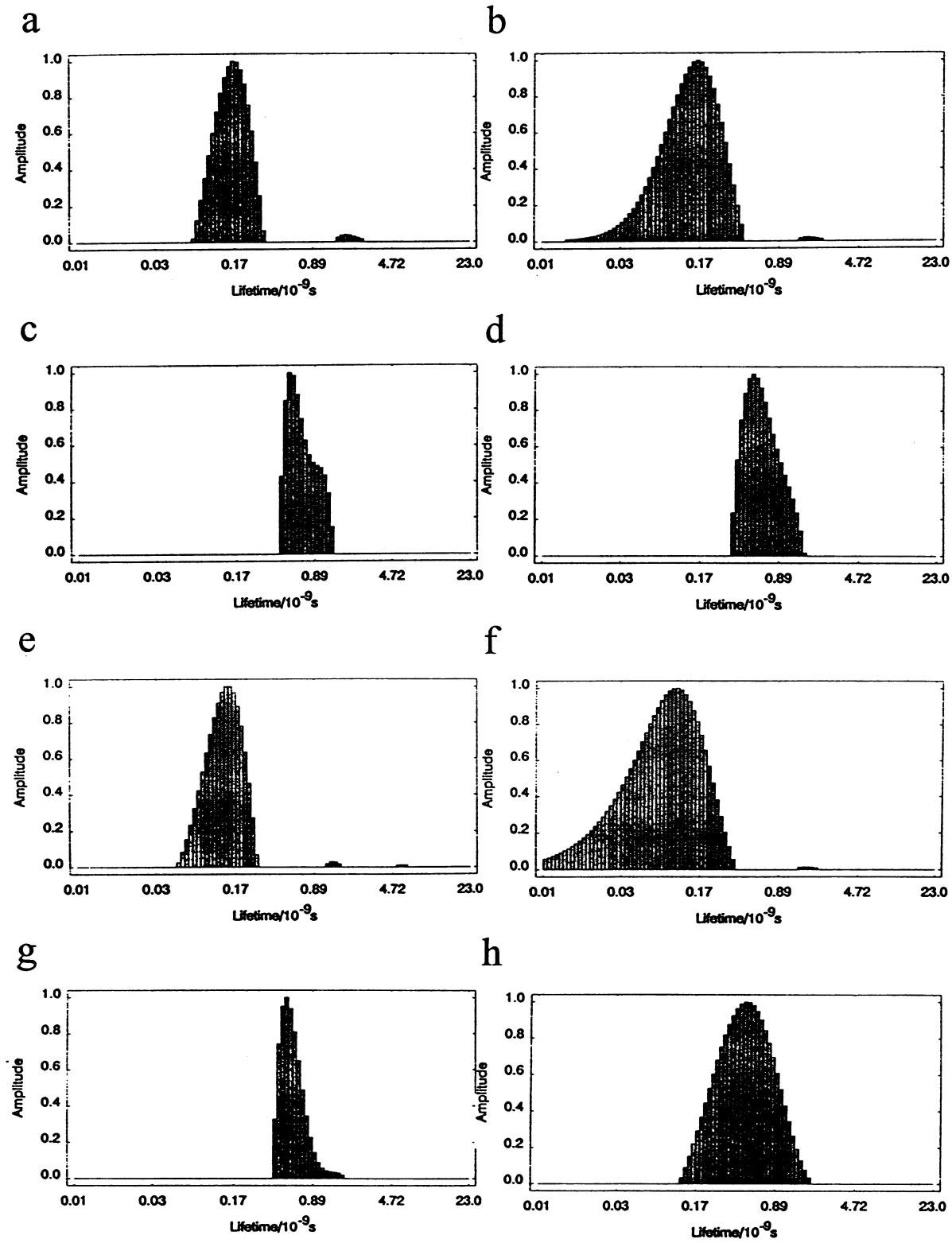

Figure 3. Lifetime distributions of the Trp fluorescence of Dns-[L-EN] (a), Dns-[D-EN] (b), F(NO_2)-[L-EN] (c), F(NO_2)-[D-EN] (d) in MeOH and Dns-[L-EN] (e), Dns-[D-EN] (f), F(NO_2)-[L-EN] (g), F(NO_2)-[D-EN] (h) in MeCN.

acids in positions 1 and 4 are highly mobile, especially in the dansyl analogues. In solvents more polar than MeOH or MeCN the number of conformational families increases. The ability of water molecules to break the intramolecular hydrogen bonds increases the conformational freedom of the main peptide chain. The narrower lifetime distribution of the dansyl containing enkephalin analogues in more polar solvents (water and Me_2SO) may be associated with the tendency of hydrophobic aromatic side chains (Dns and Trp) to sticking to the main peptide chain, which is less hydrophobic than the solvent.

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