Fluorescence molecular relaxation studies of protein dynamics

The probe binding site of melittin is rigid on the nanosecond time scale

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The methods of time-resolved nanosecond fluorescence spectroscopy, edge-excitation fluorescence shifts and the excitation wavelength dependence of the fluorescence decay were applied for the analysis of protein dipolar group relaxation dynamics in the environment of the fluorescence probe 2-(*p*-toluidinonaphthalene)-6-sulfonate bound to bee venom melittin. The results demonstrate than the internal motions in this small protein are so slow that the dipolar structural relaxation does not occur on the nanosecond time scale.

Protein dynamics *Melittin* *Fluorescence probe* *Time-resolved spectroscopy* *Edge-excitation fluorescence shift*

1. INTRODUCTION

The importance of intramolecular motions for protein functions has been widely discussed [1–3]. Molecular dynamics calculations [1,2] as well as some experimental data [4,5] suggest the existence of very fast subnanosecond mobility. Other results [6,7] show that the motions are much slower and may proceed over times of nanoseconds and microseconds.

Melittin is a major protein component of bee venom. It is a small protein of 26 residues that forms α-helical tetramers at high ionic strength. High-resolution X-ray crystallographic data demonstrate the conformational equivalence of the monomers [8]. Tetrameric melittin only binds the fluorescence probe 2,6-TNS with a dissociation constant of 4.2×10^{-6} M and short-wave shifts of the emission spectrum to 335 nm [9].

We present here results obtained from molecular relaxation spectroscopic analysis of protein dipolar group motions in the environment of the probe 2,6-TNS bound to mellitin. Our results show that these motions do not have sufficient time to occur over periods comparable to the probe excitation lifetime (nanoseconds).

Abbreviation: 2,6-TNS, 2-(*p*-toluidinonaphthalene)-6-sulfonate

2. MATERIALS AND METHODS

Mellitin was isolated from *Apis mellifera* bee venom by Sephadex G-50 gel chromatography and CM-cellulose ion-exchange chromatography [10]. Melittin was studied in 0.05 M Tris buffer (pH 7.5) + 0.15 M NaCl. Under these conditions all the preparation was in the tetrameric form, which was verified by Sephadex G-50 gel chromatography. The emission spectra and fluorescence titration behaviour correspond to those in [9]. The protein and dye concentrations were determined using molar extinction coefficients of 5570 $M^{-1} \cdot cm^{-1}$ at 280 nm for melittin and 4080 $M^{-1} \cdot cm^{-1}$ at 360 nm for 2,6-TNS. Here, 10^{-4} M 2,6-TNS and 3×10^{-4} M melittin were used. Under these conditions the dye binding is complete [9].

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Fluorescence spectra were recorded on an MPF 4 spectrofluorometer (Hitachi). The slits were 2 nm for excitation and 2–5 nm for emission.

The nanosecond decay curves and time-resolved fluorescence spectra were obtained on PRA 3000 instrumentation (Photochemical Research Associates), operating on the time-correlated single-photon counting principle. The pulse width was 2 ns, and excitation and emission slits 16 nm. Deconvolution and two-component analysis of the decay curves were performed by the computer program developed by the manufacturer.

3. RESULTS

The nanosecond time-resolved spectra of the 2,6-TNS–melittin complex are presented in fig.1. No difference is observed between early-gated and late-gated spectra; they correspond properly to time-integrated spectra. The absence of nanosecond temporal shifts indicates that the process of structural dipolar relaxation does not correlate in time with emission. In the case when relaxation proceeds in the nanosecond time range, significant time-dependent shifts are observed for 2,6-TNS in viscous solvents [11] and are associated with proteins [12]. Therefore, in the present case the characteristic range of the dipolar group intramolecular motions is beside the nanosecond time-range and may correspond to both rigid (solid-like) and mobile (liquid-like) probe environments.

In the case of fast subnanosecond mobility, the inhomogeneous broadening of spectra is dynamic and the fluorescence spectra do not depend on the excitation wavelength [7]. If the chromophore environment is rigid and the dipolar relaxational equilibrium has no time to occur, the inhomogeneous broadening of the spectra is static, and the fluorescence spectra depend on the excitation wavelength in a specific way: on the red-edge excitation they are long-wave-shifted [7]. The results presented in figs 2 and 3 show that in the case of 2,6-TNS associated with melittin the red excitation fluorescence shift is significant. On change in excitation wavelength from 360 to 400 nm the fluorescence spectra are shifted by 7–8 nm. Its tempera-

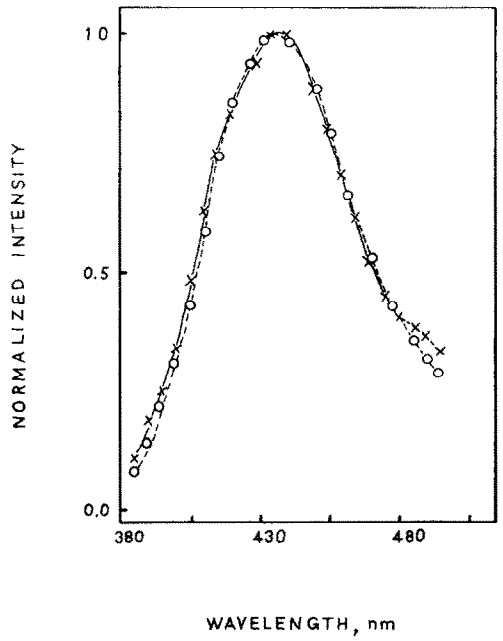

Fig.1. Time-resolved fluorescence spectra of the 2,6-TNS–melittin complex. (×—×) Early-gated spectra, channels 19–24. (○---○) Late-gated spectra, channels 55–95. 0.64 ns/channel. Excitation pulse peak at channel 19. Excitation wavelength 360 nm; 25°C.

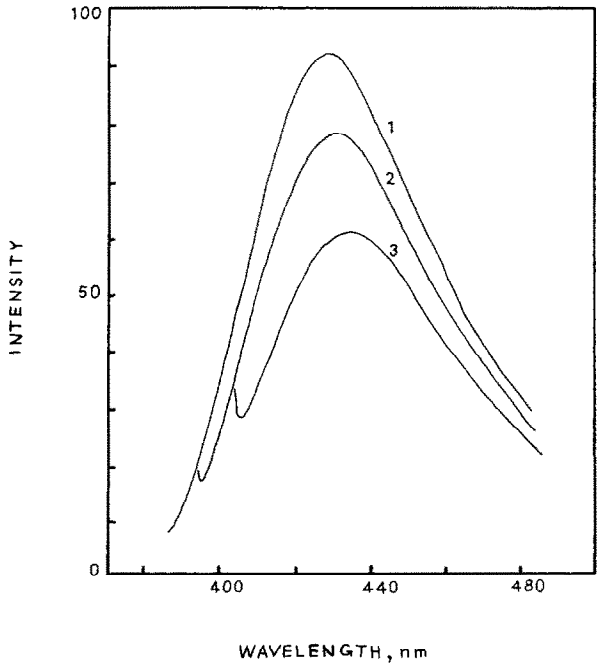

Fig.2. Steady-state fluorescence spectra of the 2,6-TNS–mellitin complex. Excitation wavelengths: 360 nm (1), 390 nm (2) and 400 nm (3).

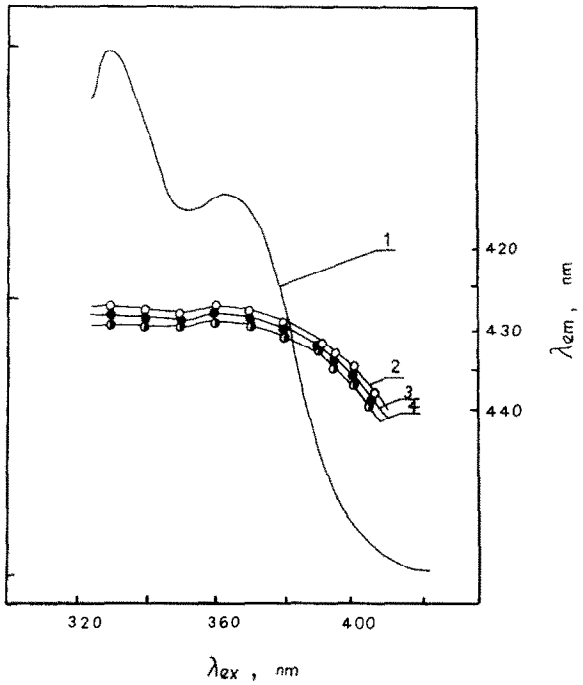

Fig.3. The excitation spectrum (1) and dependence of the maximum of the emission spectra on excitation wavelength (2-4) of the 2,6-TNS-mellitin complex. Temperature: 5°C (2), 25°C (3), 45°C (4).

ture-independent behaviour (fig.3) is additional proof of the delayed relaxation. In the case of nanosecond dynamics changes in temperature would influence the relaxation time and the magnitude of the edge excitation effect.

The results of the two-exponential analysis of the decay curves at the mean and edge excitations are presented in table 1. The excitation at the red edge results in a decrease in the excitation lifetime, and the relative intensity of the short-lived component is increased. In the case of nanosecond relaxation the already relaxed chromophores would be photoselected at the edge excitation, and the short-lived component would vanish. Thus, our data are better explained by photoselection within a population of statically broadened chromophores: for those excited at the red edge the excitation lifetime is decreased due to stronger interaction with the environment, and there is no redistribution of their interaction energy during the excitation lifetime.

Table 1

The results of two-exponential $[I(t) = A_1 \exp(-t/\tau_1) + A_2 \exp(-t/\tau_2)]$ analysis of the fluorescence decay curves of the 2,6-TNS-melittin complex at the mean (360 nm) and edge (390 nm) excitation (25°C)

λ_{ex}	Component	A	τ
360	1	0.25 (±0.03)	1.78 (±0.34)
	2	0.43 (±0.14)	9.57 (±0.15)
390	1	0.67 (±0.11)	0.58 (±0.10)
	2	0.28 (±0.06)	9.15 (±0.12)

4. DISCUSSION

Our results are important in two respects. First, they demonstrate the necessity of the complex approach based on modern molecular relaxation spectroscopy for the evaluation of dynamic information from fluorescence data. This approach includes both time-resolved and steady-state measurements under different conditions of excitation and emission. Second, they show that even for the small protein melittin the conditions of the high rigidity of conformation may be realized. On the nanosecond time scale the proteins may behave as 'micro-solids'. Therefore, further investigation is warranted to elucidate why the fast subnanosecond mobility suggested by molecular dynamics simulation [1,2] does not, at least in some cases, bring about fast relaxation of dipolar groups. Such a shift of the range of protein group mobility to slower times may be of functional importance. The protein structural dynamics becomes correlated in time and space with the elementary stages of molecular recognition and biocatalysis which proceed at times slower than nanoseconds [3].

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