

## Letter to the Editor

### Alphas and Taus of Tryptophan Fluorescence in Membranes

Two recent articles by Clayton and Sawyer in *Biophysical Journal* (1999b, 2000) address the structural and dynamic organization of class A amphipathic helical peptides at a phospholipid bilayer interface. Dynamics along the helix were probed by means of fluorescence, using a series of five single-tryptophan mutants. The results were compared with a recent structural model from our laboratory, which was obtained from x-ray structural data and molecular dynamics simulations (Hristova et al., 1999). Although the papers by Clayton and Sawyer contain interesting observations, we question their assignments of fluorescence parameters to specific molecular species or processes. Specifically, our questions concern (1) the uniqueness of the proffered interpretation of fluorescence lifetimes as being attributable to rotameric states of tryptophan; (2) the quality of data collection and analysis, which affect the apparent number of decay components; and (3) the justification of applying global analysis to an entire family of peptides. These questions arise mainly from the heterogeneous nature of the membrane interface and the experimental challenges of fluorescence measurements in membrane systems.

#### 1. Interpretation of fluorescence lifetime heterogeneity in membranes

Tryptophan fluorescence in proteins is known to exhibit a complex decay, the origins of which are not completely understood (Callis, 1999). But the intensity can nevertheless be fitted to a sum of exponentials:

$$I(t) = \sum \alpha_i \exp(-t/\tau_i)$$

The individual components are often interpreted using the rotamer hypothesis (Szabo and Rayner, 1980; Petrich et al., 1983; Laws et al., 1986), which provides a rationale for global analysis of multiple kinetics (Knutson et al., 1983). Normally, fluorescence lifetimes,  $\tau_i$ , are designated to be global (i.e., shared between the data sets), whereas pre-exponential amplitudes,  $\alpha_i$ , are designated nonglobal. In this way, the global analysis can be used to recover the spectral distribution of  $\alpha_i$  (so-called decay-associated spectra, e.g., Fig. 1) or, as applied by Clayton and Sawyer, to characterize a distribution of rotamers in a family of membrane peptides. However, the fluorescence decay heterogeneity does not have a unique interpretation (as reviewed in Ladokhin, 2000; Lakowicz, 2000). For example, dipolar relaxation of

a simple molecule in a bulk phase can produce similar-looking decay-associated spectra in the absence of any rotamers (Ladokhin, 2001; Fig. 1, right).

Numerous studies indicate that chemical heterogeneity (Gryczynski et al., 1988) and dipolar relaxation (Ladokhin, 1999, 2001; Toptygin and Brand, 2000) in indole's environment lead to complex fluorescence decay. Is it conceivable that the same phenomenon also explains fluorescence decay of membrane peptides? According to the diffraction study of our laboratory (Hristova et al., 1999), the amphipathic class A peptide (Ac-18A-amide) is located in the membrane interface (Fig. 2), a region of great thermal disorder and chemical heterogeneity (White and Wiener, 1995). The partitioning of the fluorophore into such a complex environment, rich with dipoles, must substantially influence its fluorescence, and indeed several relaxation phenomena have been observed for the fluorophores on membrane interfaces (Easter et al., 1978; Demchenko and Shcherbatska, 1985; Gakamsky et al., 1992; Ladokhin and Holloway, 1995). Therefore, the meaning of the individual decay component of membrane peptides and proteins is ambiguous, and perhaps continuous lifetime distributions would be more relevant, as suggested for chemically heterogeneous model systems (Gryczynski et al., 1988).

#### 2. Quality of data collection and analysis

One of Clayton and Sawyer's most surprising results is an apparent reduction in the number of exponentials needed to describe decay upon partitioning of peptides into membranes. We suspect, however, that inadequate data analysis and instrumentation capabilities may have impaired their

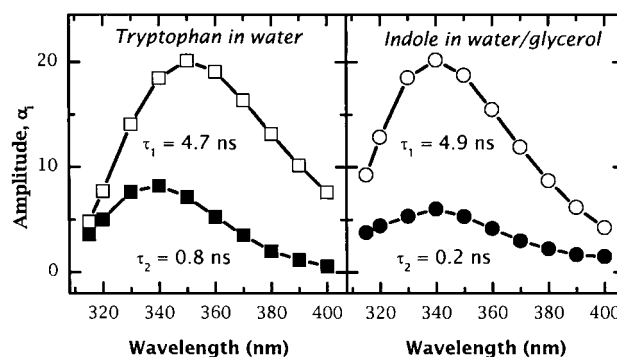


FIGURE 1 Decay-associated spectra obtained by global analysis of heterogeneous fluorescence kinetics of tryptophan in water and of indole in a water/glycerol mixture (3/2, V/V) measured at 10°C. Two components in the case of tryptophan (left) are related to rotameric forms (Szabo and Rayner, 1980). The structural relaxation in indole's environment (Ladokhin, 1999, 2001; Toptygin and Brand, 2000), in the absence of any rotamers, can lead to an apparently similar heterogeneity (right). The data are reproduced from Ladokhin, 2001.

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Address reprint requests to A. S. Ladokhin, Dept. of Physiology and Biophysics, University of California, Irvine CA 92697-4560. Tel.: 949-824-6993; Fax: 949-824-8540; E-mail: ladokhin@uci.edu

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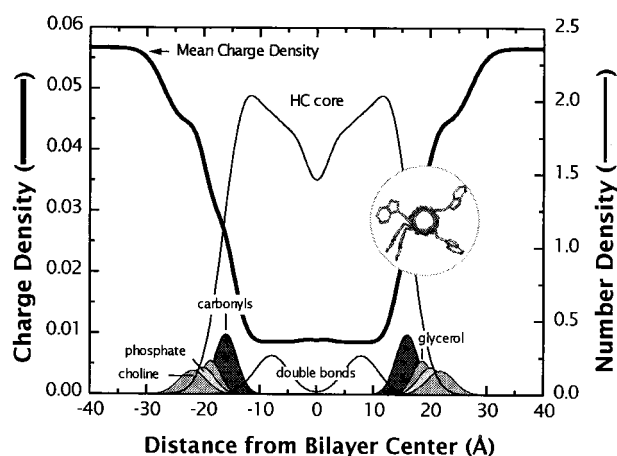


FIGURE 2 The structure of a fluid lipid bilayer and the environment of tryptophan sidechains along an amphipathic interfacial helix. The polarity profile (heavy line) was calculated by White and Wimley (1998) and based on the structure of fluid lipid bilayer by Wiener and White (1992). The various peaks represent time-averaged distributions of the principal structural groups of the lipid projected onto an axis normal to the bilayer plane. The five single tryptophan mutants of class A amphipathic peptide used by Clayton and Sawyer (1999b, 2000) are represented by the molecular model showing five sidechains sharing the same backbone. The position and size of helix are shown to scale according to Hristova et al. (1999), but the sidechain conformations are chosen arbitrarily. Clearly, the chemical structure and the overall polarity of the environment of different tryptophan sidechains varies dramatically, raising doubts concerning the validity of Clayton and Sawyer's shared lifetimes assumption (Clayton and Sawyer, 1999b, 2000).

ability to identify the shortest-lived components. Measurements of fluorescence decay of tryptophan residues in membranes, especially for the short lifetimes, are hindered by high scattering. In addition, the stray-light component allowed for in the analysis can easily mask an underresolved short lifetime. To judge the quality of the fit to their data, Clayton and Sawyer relied solely on a single parameter,  $\chi^2$ , and report only the ratio of  $\chi^2$  values for some of the models. However, to determine reliably the number of exponential components, one must use multiple criteria for goodness of fit, such as reduced  $\chi^2$ , distribution of residuals, their autocorrelation, and other criteria, reviewed in detail by Johnson (1997) and Straume et al. (1991), and illustrated in both time-domain (Peng et al., 1990; Ladokhin and Holloway, 1995; Toptygin and Brand, 2000) and frequency-domain (Gryczynski et al., 1988) fluorescence experiments.

The need for instrumentation with high temporal resolution is disclosed by studies of a simple model compound, tryptophan octyl ester. Using a flash-lamp as the excitation source on a pulse instrument with capabilities similar to the phase instrument used by Clayton and Sawyer, Chattopadhyay et al. (1997) were able to resolve only two decay components, which they attributed to rotamers. But the use of laser excitation allowed Ladokhin and Holloway (1995) to resolve three components, which were linked to the

relaxation phenomenon, based on the red-edge excitation shift. Application of a synchrotron excitation source for the same molecule (in micelles rather than bilayers) resulted in identification of four components and allowed direct observation of a relaxation-related negative pre-exponential amplitude on the red edge of emission (de Foresta et al., 1999). Therefore, we believe that Clayton and Sawyer's results probably suffered from underresolved decay kinetics.

### 3. To link or not to link: the "global" question

The Clayton and Sawyer (1999b) used a linked-lifetimes global-analysis protocol, arguing that "the lifetimes are *largely independent* of tryptophan position" (p. 3239). In our opinion, this argument alone is not sufficient because the variations in the individual lifetime components for many dissimilar proteins are not large and proper analysis requires substantial effort. The five peptides used by Clayton and Sawyer were constructed so as to place tryptophan in various positions with respect to the hydrophobic and hydrophilic faces of the helix, illustrated conceptually in Fig. 2. The tryptophans in the several peptides are likely to be in contact with different lipid chemical groups and the water environment. As a result, a strong variation (up to 15 nm) in the position of the steady-state spectra can be observed (Clayton and Sawyer, 1999a). This will influence the lifetimes of rotamers in various mutants (assuming the rotamers are the reason for the decay heterogeneity) and thus make the results of global analysis with shared lifetimes essentially meaningless.

In another example (Table 2 of Clayton and Sawyer, 2000), linking the rotational correlation times of all five peptides resulted in three trivial solutions (one of the anisotropy components equals zero), and two solutions that fit poorly (high  $\chi^2$ ). This is not surprising, as a nonlinked analysis of the same data (Table 1) indicates an over 10-fold variation in the values of two correlation times, which were inappropriately forced to be the same in subsequent global analysis.

Global analysis is a powerful technique that allows a better resolution of closely spaced parameters through simultaneous fitting of multiple sets of related data (Knutson et al., 1983). By designating certain parameters to be global (shared between the data sets), one inevitably imposes on the system (implicitly or explicitly) a physical model. Several models should be tested, and those that provide poor fits discarded. However, a good fit by itself can not be regarded as proof of a model, especially when fitting with non-orthogonal functions such as exponentials. In the latter case, multiple solutions can exist that fit data equally well (see p. 100 of Lakowicz, 1999). Also, several models could have the same set of global and nonglobal parameters (as illustrated in Fig. 1). Physical relevance should be the chief criterion for accepting a model, and vice versa; the linking of parameters should be done only with the physical model

in mind (Wang et al., 1991). For tryptophan fluorescence, we recommend two recent studies that suggest the experimental criteria for choosing among various interpretations (Ladokhin, 1999; Toptygin et al., 2001).

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Alexey S. Ladokhin and Stephen H. White

Department of Physiology and Biophysics  
University of California  
Irvine, California