Biochimica et Biophysica Acta 1838 (2014) 2289–2295

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbamem

Review

Measuring membrane penetration with depth-dependent fluorescence quenching: Distribution analysis is coming of age☆

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ARTICLE INFO

Article history:
Received 15 January 2014
Accepted 21 February 2014
Available online 1 March 2014

Keywords:
Fluorescence quenching
Membrane penetration
Distribution analysis
Parallax method
Molecular dynamics simulation

ABSTRACT

Depth-dependent fluorescence quenching by lipid-attached quenchers (e.g., bromine atoms and doxyl groups) is an important tool for determining the penetration of proteins and peptides into lipid bilayers. Extracting quantitative information and accurate calculations of the depth of the fluorophore are complicated by thermal disorder, resulting in broad distributions of the transverse positions of both quenchers and fluorophores. Twenty-one years ago a methodology called distribution analysis (DA) was introduced, based on the emerging view of the complexity of the transverse organization of lipid bilayer structure. The method is aimed at extracting quantitative information on membrane penetration, such as position and width of fluorophore's distribution along the depth coordinate and its exposure to the lipid phase. Here we review recent progress in refining the DA method and illustrate its applications to protein–membrane interactions. We demonstrate how basic assumptions of the DA approach can be validated using molecular dynamics simulations and how the precision of depth determination is improved by applying a new protocol based on a combination of steady-state and time-resolved fluorescence quenching. Using the example of the MPER fragment of the membrane-spanning domain of the HIV-1 gp41 fusion protein, we illustrate how DA applications and computer simulations can be used together to reveal the molecular organization of a protein–membrane complex. This article is part of a Special Issue entitled: Interfacially Active Peptides and Proteins. Guest Editors: William C. Wimley and Kalina Hristova.

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1. Introduction to depth-dependent fluorescence quenching

Accurate determination of the depth of membrane penetration is an important step in characterizing membrane interactions of proteins and peptides. Knowledge of the immersion depth of an intrinsic fluorophore or a site-selectively attached external probe can help elucidate membrane orientation, topology and folding of a membrane protein. Depth-dependent fluorescence quenching (DDFQ) with either bromine atoms or doxyl groups attached to lipid molecules at specific positions can be used to obtain this information. The idea behind DDFQ experiments is a simple one: the stronger the quenching observed with a particular quencher, the closer the depth of the probe to the

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http://dx.doi.org/10.1016/j.bbamem.2014.02.019
0005-2736/

depth of this quencher. In reality, estimating the exact transverse location of the fluorophore in the lipid bilayer is complicated by multiple factors, such as a limited number of available quenchers of variable depth, and deficient knowledge of the quenchers' depths. The most fundamental challenge, however, originates from the thermally disordered nature of the lipid bilayer. As a result, various lipid and protein moieties of the membrane are distributed over the ranges of depth. Thus, in order to capture the physical reality of membrane penetration, one needs to describe it in terms of distributions of depth. To answer this call within a specific application to depth-dependent fluorescence quenching, a method named distribution analysis (DA) was introduced 21 year ago [1–5]. Over this period DA evolved into a popular tool in many research labs [6–15]. Here we review recent progress in DA maturation connected to the application of advanced experimental schemes and the cross-pollination with molecular dynamics (MD) simulations, rapidly expanding into the area of membrane studies.

2. Overview of lipid-attached quenchers

The two main types of quenchers used in DDFQ experiments are bromolipids and lipids with attached spin label groups. In the early years, when mainly tryptophan fluorescence was used, bromolipids were often considered choice quenchers, because McIntosh and Holloway had accurately characterized their positions in the bilayer using X-ray diffraction [16]. The increased applications of site-selective labeling with various bright fluorescent probes caused a surge in the use of spin-labeled lipids (Fig. 1), which can quench probes that bromolipids can't (e.g., NBD and bimane). Another advantage of spin-labeled lipids is that the quenching group can also be attached to the headgroup region (TEMPO-PC) in addition to lipid acyl chains (Fig. 1), allowing examination of a shallower interfacial membrane penetration. This brought demand for a better characterization of the depth of the lipid-attached doxyl and TEMPO groups, which was achieved with various experimental approaches [17–22] and later with MD simulations [23]. Because practical applications of DDFQ require relatively high concentration of quenchers, it was important to verify that the presence of multiple bulky quenching groups does not perturb the bilayer. The recent MD simulation of a series of spin-labeled lipids has confirmed that increasing the concentration of lipid quenchers to experimentally relevant levels of about 30 M% did not affect the location of the quenching group (Fig. 2), nor the dynamic properties of the bilayer [23].

3. Principles of data analysis

In the DDFQ experiment one determines the fluorescence intensity, F, or lifetime, τ, of a probe as a function of the known depth of the quencher, h. These data are usually normalized to the corresponding values measured in the absence of quenching (F_0 and τ_0) and are used to generate a depth-dependent quenching profile, $QP(h)$, which provides visual reference of the quenching (e.g., Figs. 6b and 8). In the past, several definitions of the quenching profile were used, depending on the assumptions with regard to the nature of quenching process. The exact choice was shown to result in negligible (~0.1 Å) variations in calculated depth [24], however. Because the collisional dynamic nature of the quenching in membranes has been firmly established for both bromolipids and spin-labeled lipids [7,14,15], we suggest that the quenching profile should be defined as $QP(h) = F_0/F(h) - 1$, and as $QP(h) = \tau_0/\tau(h) - 1$, for intensity and lifetime measurements, respectively (this is further discussed in Section 6).

The DA methodology approximates the transverse quenching profile with a sum of two symmetrical Gaussian functions:

$$QP(h) = G(h) + G(-h) = \frac{S}{\sigma\sqrt{2\pi}} \exp\left[-\frac{(h-h_m)^2}{2\sigma^2}\right] + \frac{S}{\sigma\sqrt{2\pi}} \exp\left[-\frac{(h+h_m)^2}{2\sigma^2}\right] \quad (1)$$

where h_m = the center (mean) of the quenching profile, σ = the width of the distribution, and S = the area of the quenching profile. The second component, $G(-h)$, is an exact mirror image of the first one taken with respect to the bilayer center ($h = 0$) and accounts for trans-leaflet quenching in the case of the deeply penetrating fluorophores [3] (e.g., see Fig. 6). Note that either single or coupled double Gaussian fits use only three fitting parameters: h_m, σ and S, corresponding to the most probable depth of penetration, fluctuations in the transverse position, and overall accessibility to quenching (i.e., quenching efficiency), respectively.

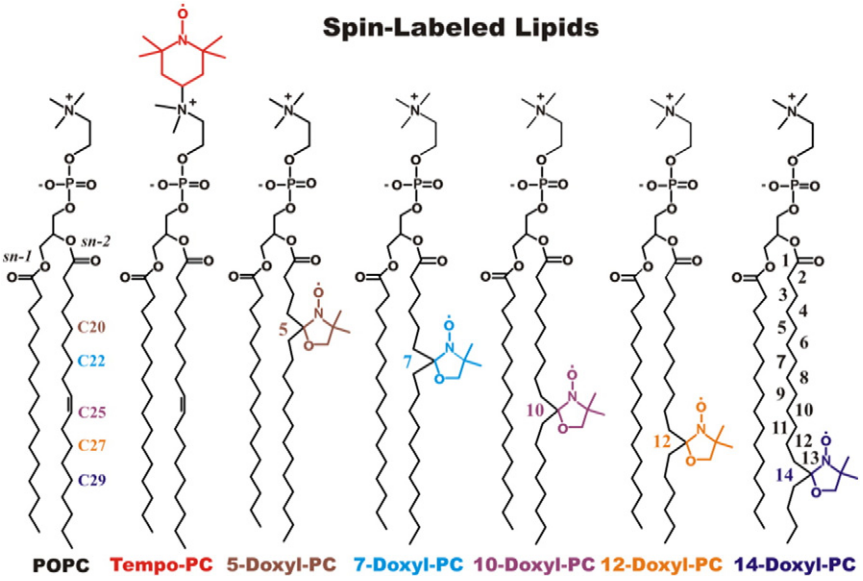

Fig. 1. Spin-labeled lipids used for depth-dependent fluorescence quenching. structure and atom numbering of unlabeled POPC, Tempo-PC (1-palmitoyl-2-oleoyl-*sn*-glycero-3-phospho(TEMPO)choline), *n*-Doxyl-PC (1-palmitoyl-2-stearoyl-(*n*-Doxyl)-*sn*-glycero-3-phosphocholine) spin-labeled lipids. A Tempo label (in red) is covalently attached to a headgroup and a Doxyl moiety (color-coded) is introduced to a variety of positions down to the sn−2 acyl chain of the host lipid. The transverse distributions and the dynamic properties of these quenchers incorporated into POPC bilayer at concentrations relevant for DDFQ experiments have been characterized by MD simulations [23].