Functional Materials 12, No.3 (2005) © 2005 — Institute for Single Crystals

A unified approach to sensitivity and specificity evaluation of novel potential fluorescent dye probes for proteins

Yu.G.Okladnoy, T.S.Dyubko[*]

National Technical University "Kharkiv Polytechnical Institute", 21 Frunze St., 61002 Kharkiv, Ukraine
[*]Institute for Cryobiology and Cryomedicine Problems, National Academy of Sciences of Ukraine, 23 Pereyaslavskaya St., 61015 Kharkiv, Ukraine

Received September 20, 2004

The existing evaluation criteria of novel fluorescent dyes proposed as potential fluorescent probes for proteins have been considered and a method has been proposed to unify the approach to the sensitivity and specificity evaluation of a new dye at interaction with a protein. The conditions of the evaluation applicability to the property selection and comparison for novel dyes proposed as potential fluorescent probes are analysed.

Рассмотрены существующие критерии оценки свойств новых флуоресцентных красителей, предлагаемых в качестве потенциальных флуоресцентных зондов для белков, и предложен способ унификации подхода к оценке чувствительности и специфичности новых красителей при взаимодействии с белком. Анализируются условия применимости данной оценки при отборе и сравнении свойств новых красителей, предлагаемых в качестве потенциальных флуоресцентных зондов.

To date, numerous fluorescent dyes have been synthesized with various spectral and luminescence properties that offer wide possibilities for the use thereof in medical and biologic investigations as fluorescent labels and probes. The fluorescent probes (FP), unlike the labels, are not bound covalently with biologic macromolecules but are in thermodynamic equilibrium state between the solvent (usually an aqueous solution) and the biologic object being studied [1, 2]. Possessing a certain affinity due to their unique chemical structure, the probes are sorbed by the biologic macromolecules at specific sites being referred to as "sorption centers" (SC). The presence of the FP molecules in the SC is due in essence only to a long time of the due molecules localization near those centers as compared to the average localization time of the molecules in the solution. The affinity of a specific FP to a specific SC is influenced by several factors, including the FP chemical nature and stereochemistry, complementation to specific binding sites of the macromolecule and the probe/protein molar ratio. It is just the latter factor that makes it possible to use the FPs as sensitive indicators of the protein conformation state and binding ability. However, to date, there are essentially no general and universal criteria to evaluate comparatively the sensitivity and specificity of those substances in relation to the biologic objects to be studied. Some progress in this way was provided by [3], but the FP sensitivity and specificity evaluation criteria proposed by that author are not universal, since those are dependent on the specific experimental conditions.

In this work, criteria are considered providing an universal evaluation of the novel fluorescent dyes proposed as potential fluorescent probes in the investigations of the protein conformation state changes. The

binding constant K characterizing quantitatively the efficiency of a FP binding with a protein molecule is among the most widely used parameters being determined when evaluating the FP interaction with proteins. The K is determined by double fluorimetric titration of the probe solution with the protein followed by that of the protein solution with the probe and construction of the Scatchard plot [1, 2]. Consideration of the K allows to evaluate also the protein binding ability (PBA). The approaches to evaluation of albumin binding ability (ABA) using FP have been elaborated in [3, 4].

To evaluate comparatively various dyes being potential FP, the following parameters are proposed to be used: (1) sensitivity index characterizing the signal intensification due to the probe binding to the protein and associated with solvatochromic properties of the dye and (2) the PBA relative to the FP being considered. These parameters can be evaluated for limiting cases (probe excess or protein excess) by varying the protein/probe ratio.

In diluted solutions, the fluorescence intensity F can be presented as [3]

$$F = 2.3I_0\varepsilon\varphi lC = ASC, \quad (1)$$

where I_0 is the exciting light intensity; ε, molar absorption coefficient of the fluorophor at the exciting wavelength; φ, the fluorescence quantum yield; l, the optical path length; C, the fluorophor concentration. This equation can be presented by the product of the instrument constant A, the parameter $S = \varepsilon\varphi$ characterizing the fluorophor absolute sensitivity and its concentration C.

In [3], the FP sensitivity has been considered to be in proportion to difference in its fluorescence with and without the protein:

$$\Delta F = F_p - F_0 = A(S_bC_b + S_fC_f) - AS_fC_0 = A(S_b - S_f)C_b, \quad (2)$$

where F_p and F_0 are the FP fluorescence intensities in the solution with and without the protein, respectively; S_f and S_b, the absolute sensitivities of the free and bound probe, respectively; C_f and C_b, the free and bound probe concentrations, respectively; $C_0 = C_f + C_b$, the probe total or initial concentration. In this case, the signal intensification due to the FP binding to the protein is in proportion to the bound probe concentration and to the value of its solvatochromic effect that can be determined from the difference between the probe fluorescent sensitivity in the free and bound states (S_b–S_f).

In practice, the fluorescence intensity is measured in conventional units, the absolute value thereof depending on the measuring instrument and conditions. To get rid of the instrument characteristics and evaluate properties of the FP itself, relative values should be considered. This can be attained by two methods:

First, by comparing the characteristics of the probe under study with those of another well-known probe, thus being used as a reference. This approach has been realized in [3] where the $\Delta F/\Delta F(ANS)$ is used as the criterion of the probe properties (that is, the ANS probe is used as the reference). If the values included in that ratio are measured using the same instrument (or two fully identical instruments) and at the same probe and the protein concentrations, then, as is shown in [3], the above ratio becomes equal to that of absolute sensitivities of the studied probe and the "reference" one. However, it is not always possible to measure using identical instruments and in identical conditions, thus, the potential of the approach is limited. The reference probe is not always available, too.

Second, to use as the probe characteristic a dimensionless ratio of the measuring results related to the specific probe only, that is independent of the instrument constant and, as far as possible, of the solution component concentrations.

To provide the comparison of the FP parameters determined in different conditions, we propose to use as the FP sensitivity characteristic the dimensionless ratio of its fluorescence intensities in the solution in the presence and absence of the protein, i.e.,

$$f_1 = F_p/F_0 = 1 + [C_b(S_b - S_f)]/S_fC_0 \quad (3)$$

and the parameter

$$f = f_1 - 1 = [C_b(S_b - S_f)]/S_fC_0, \quad (4)$$

that includes in the optimal manner the most important parameters that characterize the FP and its interaction with the protein and does not comprise the instrument constant or other parameters concerning the specific experimental conditions. For investigations and for practical purposes, of the most importance is to carry out the measurements in two limiting cases,

namely, at the excess of the protein and at that of the probe [3,4].

In the presence of excess protein, the probe acts as the marker of the protein structure state. In this case, it is possible to suppose that the probe is bound essentially completely and only one type of the protein SC (exhibiting the highest affinity) is filled. Assuming that the probe fluorescence signal reflects only the change in the binding center hydrophobicity due to the protein structure transformations [3], we get $C_b >> C_f$ and $C_b \to C_0$. In this case, the FP fluorescence intensification in the solution containing the protein is described as

$$\Delta F = A(S_b - S_f)C_0. \quad (5)$$

Accordingly, the fluorescence sensitivity of the probe as the marker of the structure state is $S_1 = S_b - S_f$ and is defined fully by the FP solvatochromic properties. The signal intensity ΔF is independent of the protein concentration and increases in parallel with the total FP concentration. The probe fluorescence is intensified also with increasing hydrophobicity of the protein binding centers in proportion with the increase of the solvatochromic effect.

The sensitivity of a specific FP can be evaluated in relation to that of the ANS FP using the ΔF measurements for all the probes in identical experimental conditions (using one and the same instrument at the same initial probe concentration C_0) [3]:

$$S_1/S_{1(ANS)} = \Delta F/\Delta F_{(ANS)}. \quad (6)$$

In the presence of excess protein, the FP is bound essentially completely, thus,

$$C_b = C_0. \quad (7)$$

In this case, the parameter f can be presented by a simple relation

$$f = (S_b - S_f)/S_f = S_1/S_f, \quad (8)$$

where $S_1 = S_b - S_f$ is the fluorescence sensitivity of the probe as the marker of the protein structure state [3].

The formulas (2) through (8) are written under assumption that the protein molecule contains the binding centers of one type only. This simplifying assumption does not effect the correctness of expressions (7) and (8), since in the presence of excess protein, only one SC type of the highest affinity is filled. But to consider the case of excess probe, the formulas (2) through (4) should be first generalized under assumption that the absolute sensitivity S_b of the bound probe will be different for different SC types. In this case, the product S_bC_b in (2)–(4) is to be replaced by the sum

$$\sum S_{bi}C_{bi},$$

where the summation is done over all the binding center types of the protein molecule. Then the formulas (2) through (4) are transformed to the from

$$\Delta F = F_p - F_0 = A(\sum S_{bi}C_{bi} + S_fC_f) - AS_fC_0 = A\sum(S_{bi} - S_f)C_{bi}, \quad (9)$$

$$F_1 = F_p/F_0 = 1 + [\sum C_{bi}(S_{bi} - S_f)]/S_fC_0, \quad (10)$$

$$f = f_1 - 1 = [\sum C_{bi}(S_{bi} - S_f)]/S_fC_0. \quad (11)$$

In conditions of an excess of the probe, the fluorescence measurement results make it possible to evaluate the PBA basing on the competition of the probes with toxic and pharmacological ligands tending to the same SC of the protein. That competition makes a base for the use of FP in diagnostics. In the presence of excess probe, all the binding centers of the protein become filled completely. For the concentration of the probe bound by the specific type of the binding center, is valid

$$C_{bi} = N_iP, \quad (12)$$

where P is the protein concentration; N_i, number of the i-th type binding centers in an individual protein molecule. Then the formulas (9) through (11) take the form

$$\Delta F = F_p - F_0 = A(P\sum N_iS_{bi} + S_fC_f) - AS_fC_0 = AP\sum(S_{bi} - S_f)N_i, \quad (13)$$

$$f_1 = F_p/F_0 = 1 + [P\sum N_i(S_{bi} - S_f)]/S_fC_0, \quad (14)$$

$$f = f_1 - 1 = [P\sum N_i(S_{bi} - S_f)]/S_fC_0. \quad (15)$$

Introducing the average value of the bound probe absolute sensitivity

$$\langle S_b \rangle = (1/N)\sum N_iS_{bi}, \quad (16)$$

where $N = \sum N_i$ is the total SC number in an individual protein molecule, the formu-

las (13) through (15) can be presented in the form

$$\Delta F = F_p - F_0 = A(NP\langle S_b\rangle + S_f C_f) - AS_f C_0 = = ANP(\langle S_b\rangle - S_f), \quad (17)$$

$$f_1 = F_p / F_0 = 1 + [NP\langle S_b\rangle - S_f)]/S_f C_0 \quad (18)$$

$$f = f_1 - 1 = NP(\langle S_b\rangle - S_f)]/S_f C_0. \quad (19)$$

The expression (17) coincides with the result from [3]. The PBA measure is the absolute sensitivity S_2

$$S_2 = N(\langle S_b\rangle - S_f) \quad (20)$$

and its ratio to the free probe sensitivity

$$S_2/S_f = N(\langle S_b\rangle - S_f)/S_f, \quad (21)$$

that, in the case of excess probe, is related to the parameter f as

$$S_2/S_f = N(\langle S_b\rangle - S_f)/S_f = fC_0/P. \quad (22)$$

In [3], it is the $S_2/S_{2(ANS)}$ ratio that is the PBA measure.

Thus, the relationships (3), (4), (8), and (22) obtained in this work make it possible to unify the approach to the sensitivity and specificity evaluation in the selection and comparison of novel dyes proposed as potential fluorescent probes for proteins.

References

1. Yu.A.Vladimirov, G.E.Dobretsov, Fluorescent Probes in Investigation of Biological Membranes, Nauka, Moscow (1980) [in Russian].
2. G.E.Dobretsov, Fluorescent Probes in Investigation of Cells, Membranes, and Lipoproteins, Nauka, Moscow (1989) [in Russian].
3. V.B.Gavrilov, *Biofizika*, **45**, 421 (2000).
4. Blood Serum Albumin in Clinical Medicine, ed. by Yu.A.Gryzunov and G.E.Dobretsov, Book 2, GEOTAR, Moscow (1998) [in Russian].

Уніфікація підходу до оцінки чутливості і специфічності нових барвників — потенційних флуоресцентних зондів для білків

Ю.Г.Окладной, Т.С.Дюбко

Розглянуто існуючі критерії оцінки властивостей нових флуоресцентних барвників, які пропонуються в якості потенційних флуоресцентних зондів для білків, і запропоновано спосіб уніфікації підходу до оцінки чутливості і специфічності нових барвників при взаємодії з білком. Аналізуються умови застосування даної оцінки при відборі і порівнянні властивостей нових барвників, які пропонуються в якості потенційних флуоресцентних зондів.