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SHORT COMMUNICATION

Fluorescence Quenching to Study Protein-ligand Binding: Common Errors

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Abstract A number of recent articles, amongst others several published in the Journal of Fluorescence, use inappropriate fluorescence methodology to determine ligand binding characteristics to (mostly) proteins. In this Letter, several common pitfalls are discussed in relation to two recent publications in the Journal of Fluorescence (Wang et al. (2009) 19:801–808; Ding et al. (2009) 19:783–791). The Author hopes that this contribution helps to prevent a further spread of the incorrect methodology, and results in a reappraisal of those articles already published using similar methodology.

Keywords Fluorescence quenching · FRET · Ligand binding · Inner-filter effect

Dear Sir,

In the last few years there has been a renewed interest in using fluorescence spectroscopy, and fluorescence quenching in particular, to characterize ligand binding to proteins. Unfortunately, a rather large number of recent papers use methodology that appears to be fundamentally flawed. This Letter is intended to point out a number of pitfalls in using fluorescence quenching to study protein-ligand binding. As examples of the possible problems in using this methodology two recently published papers in the Journal of Fluorescence (Wang et al. (Wang09) [1] and Ding et al. (Ding09) [2]) will be discussed. They contain almost all typical examples of apparently overlooked pitfalls, which are also present, to a varying extent, in previous papers published in the Journal of Fluorescence (cf. [3–14]) and a large number of other journals. I hope this Letter may serve as reference in evaluating the validity of the results in those papers.

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Ligand binding and fluorescence quenching

In order to explain the various issues, as well as to show the correct methodology, it is necessary to start with the basics of ligand binding. For a simple 1:1 ligand binding, the association (or binding) constant (K_a) is given by:

$$K_a = \frac{[PL]}{[P]*[L]} \tag{1}$$

where [PL] denotes the concentration of the protein-ligand complex, [P] the protein concentration, and [L] the ligand concentration.

When the complexation causes a change in fluorescence properties of either protein, ligand, or complex, it is in principle possible to determine the association constant. The methodology in Wang09 and Ding09 is based on this principle, and in particular on the quenching of the intrinsic tryptophan fluorescence of the protein. The mechanisms of quenching will be discussed later in this Letter.

It can be shown that if ligand binding causes the formation of a *non-fluorescent* protein-ligand complex, then the association constant is given by:

$$K_a = \frac{F_0 - F}{F*[L]} \Rightarrow \frac{F_0 - F}{F} = K_a*[L] \Rightarrow \frac{F_0}{F} = 1 + K_a*[L] \tag{2}$$

Where F_0 is the fluorescence of the protein in the absence of ligand, and F the fluorescence at a given concentration of ligand.

Equation 2 is the well-known Stern-Volmer equation, but with the Stern-Volmer constant now equal to the association constant. This equation is used by Wang09 and Ding09 to perform the initial characterization of the ligand binding, mainly to determine whether the quenching mechanism is dynamic or static.

Pitfall 1: Residual fluorescence of the complex

The formation of a complex does not always lead to a non-fluorescent complex. In fact, both Wang09 and Ding09 later discuss the quenching in terms of Förster Resonance Energy Transfer (FRET), and show that a *fluorescent* complex is formed. Unfortunately, their FRET calculations are wrong, as will be discussed later, and it is thus not possible to determine the extent of the error.

In the case of a fluorescent complex it is necessary to add a parameter to Eq. 2 which refers to fluorescence contribution by the complex:

$$\frac{F_0 - F}{F - F_c} = K_a * [L] \tag{3}$$

Where F_c is the fluorescence of the fully complexed protein.

Since F_c is the value at infinite ligand concentration, it can be calculated by fitting Eq. 3 to the experimental data. Alternatively, but less accurately, the modified Stern-Volmer equation may be used (see below) to calculate the fluorescence of the complex.

The modified Stern-Volmer equation

Ding09 also use the modified Stern-Volmer equation (Eq. 4) to study the ligand binding, but notably without explaining why they chose this equation.

$$\frac{F_0}{F_0 - F} = \frac{1}{f_a} + \frac{1}{K_a * f_a * [L]} \tag{4}$$

Where f_a is generally defined as the fraction of fluorophores that is accessible to the quencher. For a static quenching with a residual fluorescence of the complex, the factor ($1-f_a$) actually indicates the fractional fluorescence of the complex compared to the uncomplexed protein.

The modified Stern-Volmer equation has been explicitely derived for a dynamic quenching process, in which there is a constant contribution of fluorescence of the non-quenchable fraction. However, this is *not* valid for a static quenching process that results in a fluorescent complex. Nonetheless, under special conditions (see pitfall 2) the modified Stern-Volmer will yield both K_a and f_a values that come close to the true values for K_a and f_a.

Pitfall 2: free ligand versus added ligand concentration

The ligand concentration that is to be entered into Eqs. 1–4 is not the *added* ligand concentration, but rather the *free* ligand concentration. This free ligand concentration may be difficult to determine, and one thus needs to design the experiment such that $L_{free} \approx L_{added}$. The easiest method is to assure that the ligand concentration is at least a factor 10 above that of the protein concentration. However, the value of the dissociation constant ($K_d = 1/K_a$) compared to the protein concentration is also of importance to be able to measure in a proper range. In practice the method has to be designed such that the protein concentration is well below the K_d value, while the ligand concentration is close to the K_d value [15].

The potential error introduced by this pitfall is most important for the modified Stern-Volmer equation (Eq. 4), since in that equation the parameters are determined by a linear fit with the *reciprocal* ligand concentrations on the x-axis. Thus, the lowest ligand concentrations, where deviations between free and added ligand concentrations are highest, have the most influence on the linear fit.

Pitfall 3: double logarithmic plots to determine stoichiometry and binding constant

Equations 1–4 are all relevant to 1:1 complexation. Equation 1 can be modified to include ligand binding to multiple sites on the protein by introducing the stoichiometry n:

$$K_a = \frac{[PL_n]}{[P] * [L]^n} \tag{5}$$

Equations 2–4 will then also need to be modified by replacing [L] with $[L]^n$. Note that the affinity constant here is the product of n individual affinity constants $\left(\prod_{i=1}^{n} K_{a,i}\right)$.

Since n is an unknown, it is not possible to perform a linear regression using Eqs. 2–4. The simplest solution to linearize the equations is by taking the logarithm, as exemplified below using Eq. 2:

$$\frac{F_0 - F}{F} = K_a * [L]^n \Rightarrow \log \frac{F_0 - F}{F} = \log K_a + n \log [L] \tag{6}$$

While this procedure is mathematically correct, the regression depends very much on pitfall 1: assuming that a non-fluorescent complex is formed. Even a minor residual fluorescence will affect the slope of the curve, which governs both the stoichiometry (the slope itself) and the association constant. Since the latter is the power to 10 of the intercept, it is extremely sensitive to small changes in the slope of the fitting procedure. Moreover, also in Eq. 6 the previously mentioned issue of L_{free} vs L_{added} is of concern.

Ding09 have used another equation (Eq. 7a) than Wang09, which is taken from a publication by Bi et al. [16]. Note that the K_a in this equation is formally different from that of Eq. 6 and assumes equal binding sites. Ding09 unfortunately do make an error in the equation, and use Eq. 7a, rather than Eq. 7b given by Bi et al. This has little influence on the results, other than generating negative values for the stoichiometry. The derivation of Eq. 7b by Bi et al. is a bit odd, as it actually would lead more straightforward to Eq. 7c, which also allows easier plotting of the data.

$$\log\frac{F_0-F}{F} = n\log K_a + n\log\frac{1}{[L_{added}] - \frac{F_0-F}{F_0}*[P_t]} \tag{7a}$$

$$\log\frac{F_0-F}{F} = n\log K_a - n\log\frac{1}{[L_{added}] - \frac{F_0-F}{F_0}*[P_t]} \tag{7b}$$

$$\log\frac{F_0-F}{F} = n\log K_a + n \times \log\left([L_{added}] - \frac{F_0-F}{F_0}*[P_t]\right) \tag{7c}$$

Where $[P_t]$ is the total protein concentration.

Ding09 do not explain why they chose this particular equation, out of the three possible equations that Bi et al. describe. Moreover, these equations still assume a non-fluorescent complex, without providing any proof that this is the case.

Within Eq. 7 is an apparent attempt to calculate the free ligand concentration. That is:

$$[L_{free}] = [L_{added}] - \frac{F_0-F}{F_0}*[P_t] \tag{8}$$

Equation 8 can be used under two conditions; first, the complex must be non-fluorescent (pitfall 1). Second, and most importantly, Eq. 8 can only be used if the binding stoichiometry is 1:1. After all, it assumes that the amount of ligand in the complex is equal to the amount of complex itself $(((F_0-F)/F_0)*[P_t])$. This defies the whole purpose of using Eq. 7.

However, Eq. 8 can be used to get around the problems with the unknown free ligand concentration for Eq. 2, but will need to be modified for use in Eqs. 3+4:

$$[L_{free}] = [L_{added}] - \frac{F_0-F}{F_0-F_c}*[P_t] \tag{9}$$

Equations 8 and 9 also show that with $[L_{added}] >> [P_t]$, $[L_{added}] \approx [L_{free}]$, since the fluorescence term in front of P_t only varies between 0 and 1.

A final note in this section is the rather interesting problems that may arise when using Eq. 6, as exemplified by Wang09. The latter report association constants that decrease with increasing temperature for Eq. 2, but binding constants[1] that increase when using Eq. 6. The latter discrepancy is probably related to the variability in the calculated stoichiometry as a function of temperature.

The quenching mechanism

Both Wang09 and Ding09 speculate on the quenching mechanism. In principle, there are several potential causes of quenching, such as the inner-filter effect, dynamic quenching, and static quenching. The latter can be subdivided into structural changes around the fluorophore (s), ground-state complex formation, and energy transfer from the excited state of a donor to an acceptor through nonradiative dipole-dipole coupling. This last mechanism is also referred to as Förster resonance energy transfer (FRET).

Pitfall 4: the inner-filter effect

The inner-filter effect refers to the absorption of radiation going towards (excitation) or emanating from (emission) the fluorophore. That is, when an absorbing compound is added to a solution, it may reduce the amount of excitation radiation that reaches the fluorophore, or it may absorb some of the radiation emitted by the fluorophore. It is a classic pitfall, and unfortunately one that appears to dominate in Wang09 as discussed below. Also in Ding09 it may play an important role, but its influence is more difficult to determine without a re-analysis of the raw data.

The problems with the inner-filter effect become immediately apparent in Wang09 upon analysis of the absorption spectra in the range 200–400 nm (Fig. 3 in Wang09). At the lowest ligand concentration of 2 μM, the absorbance values at the excitation wavelength (280 nm) reach up to 0.15 aufs for two of the ligands (cefotaxime and ceftriaxone). The Stern-Volmer plots are measured with concentrations up to 100 μM, and thus the absorbance at the excitation wavelength ultimately will be well above 5 aufs. This strong absorbance will quench the fluorescence, simply because much less radiation is available to excite the fluorophores. The absorbance of the ligand at the emission wavelength may add to the inner-filter effect, but this is more difficult to determine from the UV spectra in Wang09.

[1] Many of the papers using this quenching methodology use the terms "association constant" and "binding constant" in a way that suggests they are considered to be two *different* constants, hence my use of these two terms here also

Ding09 show that the UV absorption at the excitation wavelength is limited, but even this limited absorption may still explain some of the observed quenching.

Although it is in principle possible to correct for the inner-filter effect, the various methods all have their limitations. Neither Ding09 nor Wang09 mention anything about any correction, and I can thus only assume that such a correction was not applied. The easiest correction method is to determine the absorbance at the excitation and emission wavelength for each concentration of ligand (including the protein without ligand), and then multiply the observed fluorescence value using the following factor [17]:

$$F_{corr} = F_{obs} * 10^{\frac{A_{exc}+A_{em}}{2}} \quad (10)$$

Where F_{corr} is the corrected fluorescence value, F_{obs} the measured fluorescence value, A_{exc} the absorption value at the excitation wavelength, and A_{em} the absorption value at the emission wavelength.

Equation 10 essentially assumes fluorescence of a point source at the center of the cuvette, a situation that is unlikely to be the case. It should also be noted that this particular equation is only valid for fluorescence spectra that are obtained in 1x1 cm cuvettes, and concomitantly, for absorbance values determine in a 1 cm cuvette. In other cuvettes the right-hand term needs to be altered to correct for the differences in pathlength. More advanced equations are also available, but all require significant validation or assumptions. Thus, the best way of dealing with the inner-filter effect is to ensure its effect is minimal. This means that concentrations must be chosen such that the absorbance of the added ligand at the excitation and emission wavelength is below 0.1.

Dynamic quenching

For the sake of discussing the methodology let us assume there is no inner-filter effect for Ding09 and Wang09. In such case the authors correctly argue that the Stern-Volmer quenching constants derived from Eq. 2 are too large to be a result of dynamic quenching. Thus, a static quenching mechanism takes place.

Pitfall 5: Static quenching due to structural changes

Ligand binding may change the conformation of the protein, and thus alter the local microenvironment around the intrinsic fluorophore(s). Both Wang09 and Ding09 argue that there are some conformational changes, which means that (part of) the observed quenching may actually be caused by these changes. This complicates some of their further analysis, where they assume FRET as the mechanism of quenching.

Pitfall 6: Static quenching due to ground-state complex formation

Ground-state complex formation generally results in a complete loss of 'native' fluorescence, due to the formation of a new electronic system with different absorption characteristics. Oddly, Wang09 claim such ground-state complex formation based on their UV spectra (Fig. 3), but the UV spectra of protein+ligand appear to be merely a superposition of those of the protein and ligand separately. Moreover, a ground-state complex cannot be analysed in the context of FRET, which Wang09 still do.

Pitfall 7: Static quenching due to an excited-state complex formation/FRET

The formation of an excited-state complex may result in the transfer of some of the excited energy of the fluorophore (the donor) to an acceptor. The ligand may act as such an acceptor, but then the fluorescence of the donor must overlap with the absorbance of the acceptor. In the case of Wang09 and Ding09 the measured absorbance values of the ligands in the relevant range are very low (often 0.02 or lower), which is close to the detection limit for most UV spectrometers. One may thus question the accuracy of the overlap calculations used to determine the Förster distance.

Again, for the sake of the argument, let us assume that the observed absorbance for the ligands is in both cases an accurate absorbance by the ligand. In that case Wang09 and Ding09 use the correct equations to determine the so-called Förster distance. The latter is the distance for a specific donor-acceptor pair where 50% of the fluorescence energy of the donor is transferred to the acceptor.

Using the Förster distance (R_0), it is possible to determine the distance between donor and acceptor (r) using Eq. 12:

$$E = 1 - \frac{F}{F_0} = \frac{R_0^6}{R_0^6 + r^6} \quad (12)$$

Importantly, Eq. 12 assumes a 1:1 complex. However, Wang09 and Ding09 appear to use (equal) concentrations of protein and ligand which are much lower than the dissociation constant. A simple calculation shows that under such conditions only a modest percentage of protein and ligand actually are in a complex; for example, with protein concentration and ligand concentration 10% of the K_d value, only 8% of the protein is actually in a complex. To assure a 1:1 complex, the ligand concentration must be much higher than that of the protein as well as higher than the K_d value. Of course, also in the FRET calculations the inner-filter effect may play a role and result in a quenching erroneously attributed to FRET. A further complication is

the frequent use of excitation at 280 nm (cf. Wang09), which also excites tyrosine residues. The complexity of the various possible FRET pairs makes proper analysis difficult, if not impossible.

The problems with the FRET calculations in Ding09 and Wang09 can also be appreciated by looking at the calculated distances between donor and acceptor. In all cases this distance is at least 3.63 nm, and up to 4.24 nm, with the Förster distance always smaller. The protein that is used, lysozyme, has a maximum end-to-end distance of 4.4 nm [18], but its main fluorescent tryptophans are located approximately in the middle. Thus, the calculated distances actually place the ligands *outside* the protein. In addition, all calculated distances are larger than the Förster distance, which means that the maximum fluorescence quenching is less than 50%. This does not correspond to several of the Stern-Volmer plots in the two papers, which indicate (far) more than 50% of quenching. Moreover, such a substantial fluorescence of the complex automatically invalidates the use of Eqs. 2, 6 and 7, which assume a non-fluorescent complex.

Conclusion

Based on two recently published papers in the Journal of Fluorescence, I have discussed a number of pitfalls that have become standard errors in analysing protein-ligand binding using fluorescence quenching. I hope that this discussion of the pitfalls will aid others in evaluating the papers on this topic and helps reduce the further spread of the faulty methodology.

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