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ARTICLE

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Limitations in linearized analyses of binding equilibria: binding of TNP-ATP to the H_4-H_5 loop of Na/K-ATPase

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Abstract Binding of TNP-ATP [2′,3′-*O*-(2,4,6-trinitrophenyl)adenosine 5′-triphosphate, a fluorescent analogue of ATP] to the K605 protein was studied. This is an isolated N-domain in the cytoplasmic loop of the Na/K-ATPase α-subunit, lying between membrane-spanning segments 4 and 5 (sequence L^{354}-I^{604}). A titration equation is derived that explicitly makes it possible to relate the fluorescence of TNP-ATP and K605 solutions to total probe concentration in the sample. Using this, it is possible to obtain the value of the dissociation constant from the titration experiment without resorting to the Scatchard plot, which is far from optimal from the statistical point of view. Using the new formula with non-linear regression analysis, a value of the dissociation constant K_D for TNP-ATP binding to the K605 protein of 3.03 ± 0.28 μM at 22 °C was obtained. A series of fits to simulated data with added noise demonstrated clearly the advantage of non-linear regression using the new formula over the commonly employed linear regression using the Scatchard plot. The procedure presented is generally applicable to binding assays using changes in the fluorescence of ligands on binding.

Keywords ATP analogue · ATPase · Non-linear fitting · Scatchard plot · Substrate binding

Introduction

ATP-binding assays with purified cytoplasmic Na/K-ATPase loops, together with mutagenesis studies of the residues thought to play a role in ATP-binding, may serve as an efficient tool in studying structure-function relationships in the catalytic site of Na/K-ATPase.

Nearly three decades ago, Hiratsuka and co-workers demonstrated that a fluorescent analogue of ATP, 2′ (or 3′)-*O*-(2,4,6-trinitrophenyl)adenosine 5′-triphosphate (further referred to as TNP-ATP) can be used for various assays of ATP binding to macromolecules (Hiratsuka 1975, 1976, 1982; Hiratsuka and Uchida 1973; Hiratsuka et al. 1973). For assays on ATP binding to Na/K-ATPase, this possibility was confirmed by Moczydlowski and Fortes (1981a, 1981b), who found that, upon binding of TNP-ATP to the enzyme, the probe fluorescence increased and the fluorescence-enhancement data were a reliable measure of TNP-ATP binding to Na/K-ATPase. According to these authors, TNP-ATP binds with high affinity and specificity to the catalytic site of the enzyme for ATP. However, the binding of pure ATP and its fluorescent analogue are not equivalent. The presence of the chromophore increases the binding energy, as indicated by the lower dissociation constant for TNP-ATP compared with that for ATP itself. In general, this effect may result from electrostatic interactions of the negative charge on the chromophore with cationic residues of the protein, as well as hydrophobic interactions of the chromophore at the binding site. Recently, Gatto and co-workers (1998) have used TNP-ATP as a competitive ligand in ATP-binding studies performed with the purified H_4-H_5 loop of Na/K-ATPase.

Analysis of ligand-binding data is a common feature in many areas of biochemical work. If a parameter proportional to the concentration of a bound ligand is obtained in a particular assay, the data involve a non-linear dependence of the observed parameter upon the total concentration of the ligand or the total concentration of the protein, whichever is varied. Popular plots for ligand binding to proteins that are designed to give a theoretical straight line (e.g. the Scatchard plot: a plot of the concentration of bound ligand divided by that of the concentration of free ligand versus bound ligand),

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though intended to render data evaluation simple, result in inherent perturbation of the error distribution of the data in the plotted points (Leatherbarrow 1990, 1991; Lin and Nielsen 1997; Peters and Pingoud 1979); Rodbard et al. 1980). Thus the direct use of such linearized plots is in severe conflict with basic assumptions that underlie the method of least-squares fitting. This fact was well understood by the originators of some of these linearized plots and is well illustrated, for example, in the contribution of Deranleau (1969), which deals also with two other frequently used linearizations: the Benesi-Hildebrand plot and the Hill plot. Nevertheless, the simple graphical means of data evaluation with neglect of data propagation and extraction of dissociation constants prevailed in real assays on ligand binding for a long time, simply because they could easily be performed without resorting to the use of a computer.

Nowadays, the potential for more sophisticated data processing has increased enormously because a moderately inexpensive present-day PC has a computational power that is more than sufficient to run professional statistics programs into which regression equations tailored to particular experimental conditions can easily be entered, if not indeed already available. Thus, the use of linearized plots has turned out to be obsolete in many respects, and a non-linear regression on the original data should be considered the method of choice, particularly when dealing with rather noisy experimental data. As noted by Leatherbarrow (1991), non-linear regression allows noisy data to be analysed without any intermediate manipulation, and thus provides results that are more likely to be statistically valid than those found with linear regression directly applied to the rearranged data of some linearized plot.

Materials and methods

Protein preparation

K605 protein, an isolated N-domain in the cytoplasmic loop of the Na/K-ATPase α-subunit between membrane-spanning segments 4 and 5 (sequence L^{354}-I^{604}), was prepared as described previously (Hofbauerova et al. 2002), and its concentration determined according to the method of Bradford (Bollag et al. 1996). The stock solution of the protein (452 μM) was kept at −20 °C in 20 mM Tris-HCl, at pH 7.5. For the measurement of TNP-ATP binding this stock solution was diluted with 50 mM Tris-HCl, pH 7.5, to a final protein concentration of either 1.6 μM for titration experiments designed to assess K_D, or to 80 μM for estimation of the γ value parameter (see below).

TNP-ATP binding

TNP-ATP (Molecular Probes, Ore., USA) was stored as a 100 μM aqueous stock solution. Aliquots of this stock solution were added to protein samples, and the samples stirred gently before fluorescence measurements were started about 2 min after adding the dye. In each titration experiment, sequential additions of TNP-ATP were made into the same cuvettes, and the concentration of the protein was corrected for volume dilution.

Fluorescence measurements

The fluorescence intensity of protein solutions stained with TNP-ATP was measured in a FluoroMax-2 spectrofluorimeter (Jobin Yvon/Spex), in 0.4×1 cm cross-section quartz cuvettes at room temperature (22 °C). The excitation and emission wavelengths were 462 nm and 527 nm, respectively. The band passes were set at 10 nm in both the excitation and emission. Measured fluorescence intensities were corrected for blank signals due to both protein emission and scattered excitation light in the protein-containing samples, the latter only in the ligand-free samples. A flattening of the titration curve due to inner filter effects was moderate in our titration experiments (about 2% at 4 μM TNP-ATP, and practically negligible below 2 μM TNP-ATP) and was neglected in further processing of the experimental data.

Estimation of the γ value

The value of the fluorescence intensity enhancement factor γ (see Eq. 6 in the Theory section) was assessed using the method described in Appendix B, i.e., by comparing the slopes in plots of fluorescence intensity versus TNP-ATP concentration in protein-free samples with those found in samples containing the concentrated solution of the K605 protein (80 μM), in which essentially all of the fluorophore is bound.

Data fitting

The dissociation constant that characterizes the binding of TNP-ATP to K605 was assessed by fitting experimental data to the titration formulas described in the Theory section, i.e., to Eqs. (10) and (11). Data fitting was carried out using a non-linear least-squares method available in the fitting utilities of Origin 6.0 (Microcal Software, USA). The values of the parameter γ and of the protein concentration were both fixed during the fitting procedure.

Theory

Binding of small fluorescent molecules to an enzyme

We will present here a theoretical treatment that is relevant for the most simple situation, that for homogeneous ligands which bind to a homogeneous population of proteins that contain one binding site per protein, and its extension to proteins with multiple, non-interaction identical sites. Moreover, we will focus on the particular case of fluorescent ligands that exhibit a change in fluorescence response upon binding to macromolecules (as is the case of TNP-ATP binding to various macromolecules).

The solution to the TNP-ATP binding problem is relatively simple and starts from the well-known equilibrium equation for the one-site binding situation:

$$K_D = \frac{[L][P]}{[PL]} \quad (1)$$

where [L] is the concentration of free ligand, [P] is the concentration of free protein and [PL] is the concentration of probe bound to the protein. Obviously, the above concentrations are related to the total

concentrations of probe and enzyme in the sample ($[L]_T$ and $[P]_T$, respectively) by:

$$[L]_T = [L] + [PL] \quad (2)$$

$$[P]_T = [P] + [PL] \quad (3)$$

The overall fluorescence intensity, F, of the protein solution to which a fluorescent probe has been added consists of two emission components: fluorescence from probe molecules free in aqueous solution and fluorescence from probe molecules bound to the protein. If we denote their respective intensities as F_f and F_b, we can write:

$$F = F_f + F_b \quad (4)$$

Under experimental conditions typical of binding experiments, we can assume that the intensity of fluorescence emitted by a particular fluorophore in a particular situation is proportional to its concentration. Hence we can write for the emission of the free probe:

$$F_f = Q[L] \quad (5)$$

where Q is a multiplicative constant that is the product of two factors: (1) the fluorescence quantum yield of the free probe, and (2) an instrumental constant that quantifies the performance of the fluorimeter system convoluted with the properties of the free and bound fluorophore.

If the ratio of the respective fluorescence responses of bound to free probe is denoted as γ (irrespective of whether it increases or decreases), we may express the observed intensity of bound probe fluorescence as:

$$F_b = \gamma Q[PL] \quad (6)$$

Substituting Eqs. (2), (5) and (6) into Eq. (4) we obtain:

$$[PL] = \frac{(F/Q) - [L]_T}{\gamma - 1} \quad (7)$$

Up to Eq. (7), the derivation is practically identical with that of Moczydlowski and Fortes (1981a, 1981b), except that n non-interacting binding sites per protein unit were allowed for their derivation, and who then proceeded to the usual linearized Scatchard plot relationship:

$$\frac{[PL]}{[L][P]_T} = \frac{1}{K_D}\left(n - \frac{[PL]}{[P]_T}\right) \quad (8)$$

We decided to proceed in a different way, and to derive instead an explicit titration formula for F, i.e., to present F as an explicit function of $[L]_T$, which is the direct variable in real titration experiments. In order to avoid a clumsiness in setting the final formulas, we will denote the ratio (F/Q) as F^* which has, obviously, the dimension of probe concentration. We can than combine Eqs. (1), (2), (3) and (7) to obtain the following expression for K_D:

$$K_D = \frac{\left(\gamma[L]_T - F^*\right)\left([P]_T - \frac{F^* - [L]_T}{\gamma - 1}\right)}{\left(F^* - [L]_T\right)} \quad (9)$$

With a little algebra, this formula can be rearranged to produce a quadratic equation for the normalized fluorescence intensity F^* (see Appendix A). The final formula in which the experimental quantity F^* is presented as function of total concentrations $[L]_T$ and $[P]_T$ is:

$$F^* = [L]_T + \frac{\gamma - 1}{2}\left([L]_T + [P]_T + K_D - \sqrt{\left([L]_T + [P]_T + K_D\right)^2 - 4[L]_T[P]_T}\right) \quad (10)$$

This formula represents a desirable fitting equation for experimental data from titration experiments, and thus for K_D assessment, via non-linear regression. Note that it is easily extended to systems of proteins with n identical, non-interacting, non-cooperative binding sites per protein molecule, for which:

$$F^* = [L]_T + \frac{\gamma - 1}{2}\left([L]_T + n[P]_T + K_D - \sqrt{\left([L]_T + n[P]_T + K_D\right)^2 - 4n[L]_T[P]_T}\right) \quad (11)$$

In principle, one could similarly derive more complex ligand-binding formulas that would include the existence of several binding sites with different K_D, possible positively or negatively cooperative interactions between binding sites, or binding of multiple different ligands to the same binding site (Johnson and Straume, 2000). The derivation of an explicit equation for apparent probe fluorescence intensity is rather complicated for any of the above situations, and beyond the scope of the present contribution. Nevertheless, the case of competitive binding of ATP and its fluorescent analogue is of particular interest and will be discussed in a subsequent communication (M. Kubala et al., in preparation).

Results and discussion

Assessment of K_D for TNP-ATP binding to K605 protein

We measured the binding of TNP-ATP to the K605 protein, as described under Materials and methods. Typical results of titration experiments are shown in Fig. 1a. The Scatchard plot of the same data is presented in Fig. 1b.

Before fitting Eqs. (8) and (10) to the experimental data, we assessed the ratio of respective fluorescence

responses for bound and free TNP-ATP following the procedure described in Appendix B. For this purpose we used 80 μM solutions of K605. This concentration is high enough, compared with the estimated values of K_D, to satisfy the theoretical assumptions underlying the method. For the emission and excitation wavelengths used in this study we obtained $\gamma = 7.0 \pm 0.7$, and introduced the constant value $\gamma = 7$ into both fitting equations.

When we fitted the experimental data of Fig. 1a to Eq. (11) with n as well as K_D a free parameter, we obtained $K_D = 4.03 \pm 1.14$ μM and the number of binding sites per protein $n = 1.12 \pm 0.20$. Within experimental error, then, the value of n obtained corresponds identically with the presence of a single TNP-ATP binding site per protein. We therefore repeated the regression with n fixed to unity, i.e., using Eq. (10). Such a reduction in the number of parameters to be determined by the fitting procedure is expected to improve the precision of the assessment of K_D, and with the latter fitting procedure we duly obtained $K_D = 3.36 \pm 0.11$ μM. Linear regression on the Scatchard plot of the same data, again with n fixed at unity, resulted in $K_D = 3.75 \pm 0.31$ μM. In view of the expected effects of noise propagation in rearranged data in Scatchard plots, the difference between these two assessments of K_D (approximately 12%) is not surprising. This conclusion will be supported further by the results of the analysis of simulated data presented below.

Improving the signal-to-noise ratio in the experimental data could obviously reduce the difference between the results of the two regression procedures: in the case of ideal (i.e., noiseless) data the results must be identical. A desirable improvement in signal-to-noise ratio can, in principle, be achieved by averaging the data from several experiments. In Fig. 2a and b, a relatively smooth titration curve obtained by averaging the results of five titration experiments and its Scatchard counterpart are shown. Non-linear unweighted regression with n fixed to unity gave $K_D = 3.02 \pm 0.04$ μM for the former,

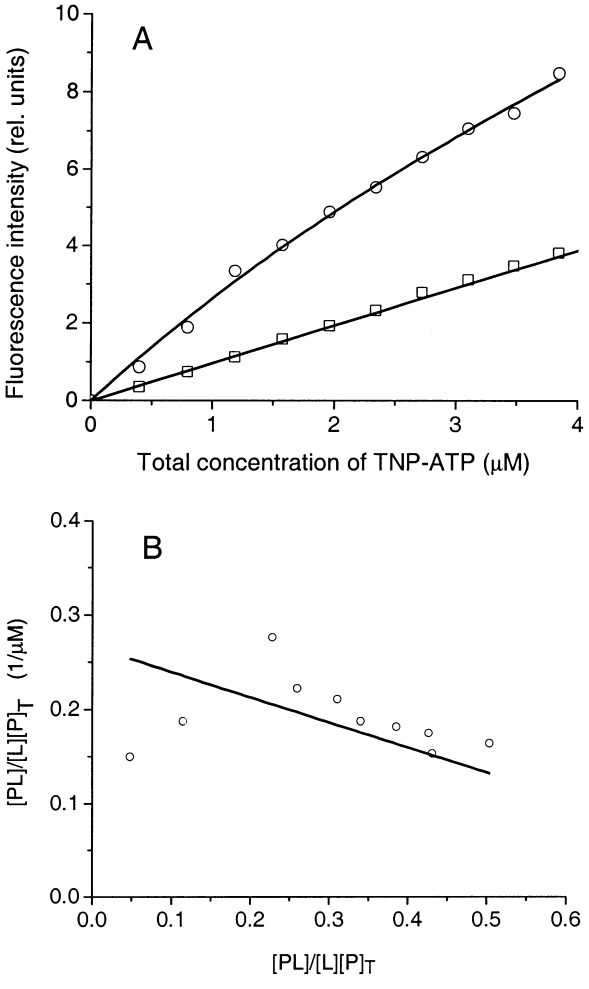

Fig. 1A, B Fluorescence titration of K605 protein with TNP-ATP. Sequential additions of TNP-ATP were made into cuvettes containing solutions with (*circles*) or without (*squares*) protein. The protein concentration was 1.6 μM in 50 mM Tris-HCl, pH 7.5. **A** Direct titration plot; **B** Scatchard plot

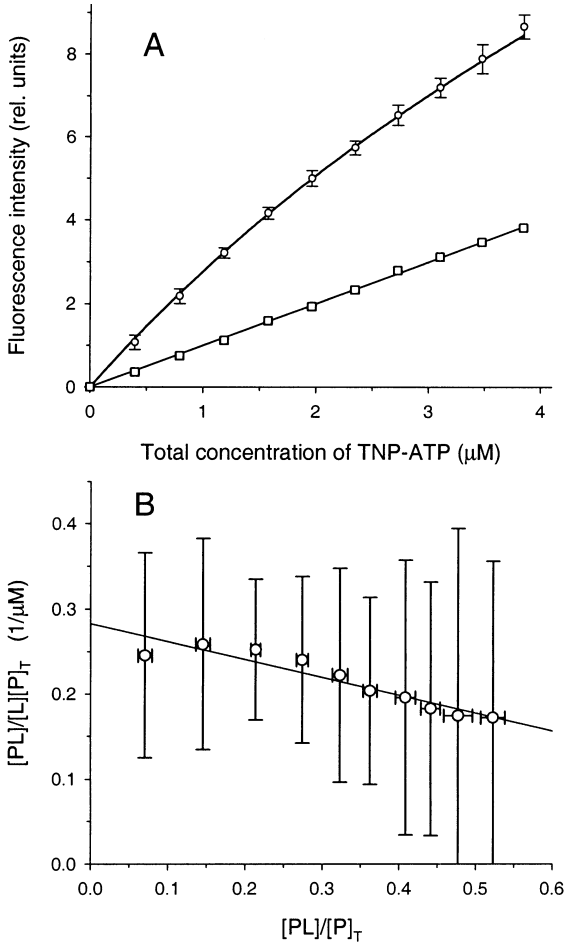

Fig. 2A, B Fluorescence titration of K605 protein with TNP-ATP: the effect of data averaging. The titration experiment performed under conditions identical to that of Fig. 1 was repeated five times, the average fluorescence intensity calculated for each TNP-ATP concentration, and the results used to make this figure. **A** Direct titration plot; **B** Scatchard plot

while linear regression on a Scatchard plot yielded $K_D = 3.53 \pm 0.12$ μM.

For all data points of the titration curve the variances were calculated (the error bars in Fig. 2a represent corresponding standard deviations). These variances were used to perform a weighted non-linear regression with titration Eq. (10), yielding $K_D = 3.06 \pm 0.12$ μM. To learn how the error of the F^* measurement propagated to the Scatchard plot data points, we have used a Taylor approximation for the standard deviations of $[PL]/[P]_T$ and $[PL]/[L][P]_T$. Assuming that the errors in $[P]_T$ and $[L]_T$ are small compared to the error in F^*, the variance in the result of a data transformation $x = x(F^*, [P]_T, [L]_T)$ can be practically determined by the quantity:

$$\sigma_x = \frac{\partial x}{\partial F^*}\sigma_{F^*} \tag{12}$$

as shown, for example, in Bevington (1969). The propagated errors that resulted from the data of Fig. 2a are shown in Fig. 2b. A fit to the linearized data that included propagated errors in both coordinates was also performed using free software available at http://bardeen.physics.csbsju.edu/stats/XYFP_NROW_form.html. The latter linear regression yielded $K_D = 3.48 \pm 0.55$ μM. The K_D values obtained with weighted fits are very close to the results of the unweighted approach, but their standard errors considerably larger, which indicates that the real uncertainty of K_D assessments may be higher than the results of unweighted regressions suggests.

To illustrate clearly that, for noisy binding data, their fitting to Eq. (10) is superior to the use of the Scatchard plot, and thus K_D values obtained in this way are more reliable, we performed a series of fits with simulated data to which computer-generated noise was added. The main goal of this paper is to introduce the new titration equation that explicitly relates the fluorescence of TNP-ATP in K605 protein solutions to the total probe concentration in the sample. Since the published assays on the TNP-ATP binding to proteins employed Scatchard plots, we focused the following simulations and related discussion on this particular linearization only.

Comparison of titration equation and Scatchard fits in processing simulated noisy data

A series of regression analyses was performed with simulated noisy titration data with a known level of Gaussian noise. We processed the simulated titration curves by the unweighted regression only because this approach is typical of routine titration experiments, in which the number of repeated runs is usually not high enough to obtain reasonable variances of individual data points.

Assuming a protein with a single TNP-ATP-binding site of $K_D = 3.0$ μM, we calculated an ideal F^* versus $[L]_T$ titration curve from Eq. (10), with the parameters γ and $[P]_T$ set to $\gamma = 7.0$ and to $[P]_T = 1.6$ μM, respectively. The data were calculated for finite steps in the variable $[L]_T$ equal to those shown in Figs. 1a and 2a. Gaussian-distributed noise with a zero mean and standard deviation of 1.0 was generated using a standard Gaussian noise generator. Simulations of noisy titration curves with different signal (i.e., fluorescence)-to-noise ratios were performed in two different ways: (1) with an additive noise expected for a small change in probe fluorescence on top of a large background, and (2) with a multiplicative noise that is a typical attribute of the fluorescence intensity measurement itself.

In the former case, for every F^* value calculated as described above, a random noise value was obtained using the Gaussian noise generator and multiplied by the mean F^* value in the $[L]_T = 0.4$–4.0 μM interval (i.e., by $F^* = 5.4$). This product was multiplied further by a factor defining the noise-to-fluorescence ratio (i.e., 0.03, 0.05, 0.10 and 0.15) and the result finally added to F^*. In the latter case, the actual value of F^* instead of $F^* = 5.4$ was used to multiply the product of the noise generator. In Table 1, all data presented in a single row were obtained with an identical result of noise generation. The simulation was repeated three times for each of the noise levels and type of noise.

The noisy simulated data sets were fitted both by non-linear regression to the titration equation (Eq. 10) and by linear regression to the derived Scatchard plot. In both cases, n was fixed at $n = 1$. The results of such fits are summarized in Table 1. While fitting with the titration equation (Eq. 10) led to acceptable results even at the highest noise-to-fluorescence ratio of 0.15 (contribution of 15%), the treatment of binding data with the Scatchard plot became unreliable much earlier, especially in the case of the additive noise.

These results support the position stated in the Introduction, i.e., whenever it is possible to construct an explicit formula that describes the amount of bound ligand as a function of $[L]_T$, the Scatchard plot and linear regression should be avoided in favour of direct non-linear regression on the titration curve. However, as pointed out by Peters and Pingoud (1979), Scatchard analysis can be critical for the evaluation of binding experiments when the binding model has not been established. In situations when no reliable information on the number and homogeneity of binding sites is available, a simple examination of the Scatchard plot, at least for data exhibiting low enough noise levels, may either support or exclude the use of the titration formula for simple single- or multiple-site binding.

In conclusion, the main result of this work is the derivation of explicit titration formulas that describe the intensity of fluorescent ligand emission in the case of single-site binding and simple multiple-site binding to a protein as a function of the total concentrations of both the protein and the ligand. With a series of computer simulations of TNP-ATP binding to K605 protein, it was demonstrated that assessment of the dissociation constant with these explicit formulas and non-linear regression is superior to the use of the Scatchard plot with linear regression.

Table 1 TNP-ATP binding to K605 protein: regressions on simulated data[a]

Noise level	Multiplicative noise				Additive noise			
	Titration fit (μM)		Scatchard plot (μM)		Titration fit (μM)		Scatchard plot (μM)	
3%	2.75 ± 0.08	2.90 ± 0.08	3.19 ± 0.14	3.35 ± 0.13	3.08 ± 0.12	2.73 ± 0.19	2.87 ± 0.07	2.91 ± 0.11	2.91 ± 0.12	2.58 ± 0.13	1.87 ± 0.34	2.66 ± 0.21
5%	2.70 ± 0.12	3.36 ± 0.22	3.18 ± 0.18	3.11 ± 0.26	3.32 ± 0.22	2.98 ± 0.35	2.90 ± 0.16	2.87 ± 0.15	3.12 ± 0.12	4.00 ± 0.41	4.11 ± 0.72	3.75 ± 0.50
10%	3.42 ± 0.25	2.54 ± 0.29	2.69 ± 0.37	3.41 ± 0.28	3.54 ± 0.49	4.95 ± 0.95	3.36 ± 0.18	2.66 ± 0.22	2.84 ± 0.33	3.92 ± 0.30	3.19 ± 0.56	8.62 ± 2.22
15%	2.05 ± 0.35	3.48 ± 0.53	3.09 ± 0.68	3.34 ± 1.00	4.55 ± 1.59	3.55 ± 1.20	3.40 ± 0.70	3.28 ± 0.58	2.20 ± 0.35	4.54 ± 5.57	4.87 ± 2.60	1.45 ± 0.19

[a]Model data were calculated for $K_D = 3$ μM, $[P]_T = 1.6$ μM, $\gamma = 7$, and $[L]_T$ ranging from 0.4 to 4 μM in steps identical to real titration experiments; computer-generated noise was added as described under Results

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Appendix

A: normalized fluorescence intensity

On multiplication of both sides by $(\gamma-1)(F^*-[L]_T)$, Eq. (9) becomes:

$$(\gamma-1)K_D(F^* - K_D[L]_T) = (\gamma[L]_T - F^*)\left\{(\gamma-1)[P]_T - F^* + [L]_T\right\} \quad \text{(A1)}$$

hence the final quadratic equation:

$$(F^* - [L]_T)^2 - (\gamma-1)([L]_T + [P]_T + K_D)(F^* - [L]_T) + (\gamma-1)^2[L]_T[P]_T = 0 \quad \text{(A2)}$$

is usefully obtained, leading to:

$$F^*_{1,2} = [L]_T + \frac{\gamma-1}{2}\left([L]_T + [P]_T + K_D \pm \sqrt{([L]_T + [P]_T + K_D)^2 - 4[L]_T[P]_T}\right) \quad \text{(A3)}$$

One can easily verify that it is the ''minus square-root'' alternative that satisfies the initial condition $F^* = 0$ for $[L]_T = 0$, as well as the result of titration experiments performed in the absence of protein, i.e., $F^* = [L]_T$ for $[P]_T = 0$.

B: assessment of the γ factor

On substituting $[P]_T = 0$ into Eq. (A3), i.e., for protein-free samples, the normalized fluorescence intensity F^* equates with the ligand concentration:

$$F^* = [L]_T \quad \text{(B1)}$$

The other limiting situation can be reached with samples of very high protein concentration, such that $[P]_T >> K_D$. In the initial phase of a titration experiment performed with such a sample, when both $[L]_T$ and [PL] are very low compared to $[P]_T$, the ratio of occupied binding sites is negligible, i.e. $[P] \approx [P]_T$. The equilibrium of Eq. (1) then leads to the following conclusion:

$$\frac{[L]}{[PL]} = \frac{K_D}{[P]_T} << 1 \quad \text{(B2)}$$

which is equivalent to the statement that practically all ligands are present in the bound form. The intensity of fluorescence from samples containing protein at high concentration is thus given by:

$$F^* = \gamma[\mathrm{L}]_\mathrm{T} \qquad \text{(B3)}$$

The value of the γ factor is thus obtained as the ratio of initial slopes in F^* versus $[\mathrm{P}]_\mathrm{T}$ plots that have been obtained for a sample containing concentrated protein and no protein, respectively.

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