Chapter 2

Steady-State Fluorescence Polarization/Anisotropy for the Study of Protein Interactions

Nicholas G. James and David M. Jameson

Abstract

Fluorescence methods are often employed for the characterization of molecular interactions. In particular, polarization/anisotropy studies are widely utilized in the life sciences as they allow quantification of protein interactions in the micro- and nanomolar concentration range. Herein we shall briefly describe the theoretical aspects of polarization/anisotropy and outline an experiment for determination of the dissociation constant for a protein–ligand complex.

Key words Polarization, Anisotropy, Protein interaction, Protein–ligand binding

1 Introduction

Fluorescence methodologies have been used in the biological and biochemical fields since the first half of the last century [1, 2]. Fluorescence studies offer unique advantages over traditional biochemical approaches to ligand–protein interactions, such as column chromatography or radiolabeling, by providing information down to subnanomolar concentrations. The characterization and understanding of the forces driving these interactions are of critical importance since biological specificity, essential to life, is largely based on protein–ligand interactions. In many cases, the intrinsic protein fluorescence, largely due to tryptophan and to a lesser extent tyrosine residues [3], can be used to monitor the protein interaction. In other cases, an extrinsic fluorescence probe can be attached to the protein or ligand. Although direct intensity measurements can be valuable for detecting protein interactions, in some cases, the fluorescence intensity change upon ligand binding is negligible [4, 5]. In these cases, protein interactions can often be monitored using polarization, which relies on the differences in rotational mobility of bound vs. free ligand; hence a change in fluorescence yield or spectral shift is not required. Polarization studies have

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been used by biochemists for studying protein interactions for more than 50 years as the theory had been thoroughly developed and tested by Francis Perrin and Gregorio Weber [6, 7]. However, the introduction of the TDx polarization instrument for fluorescence immunoassays by Abbott Laboratories in the early 1980s [8] as well as the development of polarization plate readers soon thereafter significantly increased the use of this method because it introduced a broader community to the rapid screening of a large number of protein–ligand combinations. Polarization has since become one of the most prominent methods for investigating protein interaction as polarization measurements are done at equilibrium (no need to separate bound and free) and can be easily automated [9].

Herein we demonstrate how to use fluorescence polarization to determine the fraction of bound and free ligand when monitoring protein–ligand interaction. We start with a large excess (relative to the K_d) of the species (protein or ligand) that is not being monitored, such that all of the fluorescent ligand or protein is bound. Successive dilution of the sample not being monitored (e.g., ligand), while maintaining the concentration of the monitored substance (e.g., protein), provides a complete binding isotherm. In case the emission intensity changes during the outlined experiment, this change is taken into account, as outlined in the theory section, when determining the fraction bound. We note that in the case of significant intensity changes, there is no need to measure fluorescence polarization/anisotropy to follow the binding. But we also note that intensity is an "extensive property," i.e., it depends on the amount of material present, while polarization/anisotropy is an "intensive property," which is independent of the amount of material (the fluorescence lifetime is also an intensive parameter). There are often advantages to the use of intensive parameters, for example, the fact that they are independent of the instrument platform being used for the measurements. From the plot of fraction bound and protein concentration, we quantify the dissociation constant (K_d) for this protein–ligand pair. An example, from the literature, is also provided in which the polarization change of the ligand is monitored, as well as an example determining the strength of protein–protein interaction [10, 11]. A similar procedure, along with a rigorous treatment of attendant errors, has been described previously [12] and has been used to determine dissociation constants in a number of reports [13, 14].

1.1 Theory

For a molecule to fluoresce, an energy source capable of promoting the molecule to an excited state must be utilized. The most common way of achieving these specific energies is through a xenon arc lamp and a monochromator, which allows for the selection of wavelengths in the range of 200–1,000 nm. To achieve polarized emission from the sample, a specific electric vector of the excitation

light must be isolated as the excitation source typically has electronic radiation aligned in all directions within the plane perpendicular to the propagation of the light. This isolation is traditionally done by placing a polarizer orientated parallel to the laboratory axis just after the light source. Such polarization of light allows for the specific excitation of fluorophores that have excitation vectors aligned properly with the plane-polarized excitation light. This phenomenon is termed photoselection. The polarization (P) is then determined by measuring emission through a polarizer parallel ($I_{\|}$) and perpendicular ($I_{\perp}$) to the excitation light:

$$P = \left(\frac{I_{\|} - I_{\perp}}{I_{\|} + I_{\perp}}\right) \tag{1}$$

Another frequently used expression, in the context of polarization, is anisotropy and is defined as:

$$r = \left(\frac{I_{\|} - I_{\perp}}{I_{\|} + 2I_{\perp}}\right) \tag{2}$$

The information content from polarization and anisotropy is essentially identical [15] and can be interconverted using the expression:

$$r = \frac{2P}{(3 - P)} \tag{3}$$

Some researchers fervently adhere to the use of the polarization function, while others cling just as fervently to the anisotropy function. We simply note that anisotropy is probably the more popular function in biophysical studies, while polarization is almost universally used in clinical chemistry application. The limits of polarization, in a completely orientated system like a crystal, are +1 or −1 (+1 or −0.5 for anisotropy) as defined by the equations above. However, in the case of a solution of fluorophores, these theoretical limits are not reached. This partial depolarization is due to the random orientation of the electric vector of each molecule in an ensemble of randomly oriented fluorophores. The limits for these cases have been derived, based on photoselection rules, as +0.5 and −0.33 for polarization or +0.4 and −0.2 for anisotropy [7]. A number of factors contribute to the observed polarization of a fluorophore. One of the factors, which is a measure of polarization of the fluorophore in the absence of rotation, is termed the limiting polarization (P_0) and is determined:

$$\frac{1}{P_0} - \frac{1}{3} = \frac{5}{3}\left(\frac{2}{3\cos^2\theta - 1}\right) \tag{4}$$

where θ is the angle between absorption and emission oscillator.

Polarization measures the rotational mobility of the fluorescent molecule, which makes it more appealing for monitoring

interactions than intensity measurements. In 1925–1926, Francis Perrin derived an expression for obtaining the polarization based on the rotational diffusion and excited state lifetime [6]:

$$\frac{1}{P}-\frac{1}{3}=\left(\frac{1}{P_0}-\frac{1}{3}\right)\left(1+\left(\frac{RT}{\eta V}\right)\tau\right) \tag{5}$$

where P is the polarization observed, P_0 is the limiting polarization of the fluorophore, R is the gas constant, T is the absolute temperature, V is the molar volume of the fluorescent molecule, η is the solvent viscosity, and τ is the excited state lifetime. This equation is often rewritten to contain the Debye rotational relaxation time (ρ):

$$\frac{1}{P}-\frac{1}{3}=\left(\frac{1}{P_0}-\frac{1}{3}\right)\left(1+\frac{3\tau}{\rho}\right) \tag{6}$$

The major advantage of polarization, compared to the other methods, is its ability to quantify the extent of bound ligand in a sample without recourse to a separation step, as, for example, is required with radioisotope labeling. This mathematical resolution of free from bound is accomplished through the additivity of polarization of different species, which was derived by Gregorio Weber in 1952 [7]:

$$\left(\frac{1}{P_{\text{obs}}}-\frac{1}{3}\right)^{-1}=\sum f_i\left(\frac{1}{P_i}-\frac{1}{3}\right)^{-1} \tag{7}$$

where P_{obs} is the actual observed polarization coming from all the components, f_i is the fractional contribution of the ith component to the photocurrent, and P_i is the polarization of the ith component. Additivity for anisotropy can also be expressed as:

$$r_{\text{obs}}=\sum f_i r_i \tag{8}$$

As can be deduced from the previous equations, a change in fractional contribution to the photocurrent of the bound or free fluorophore during the experiment will alter their contributions in the additivity equation. This bias can be corrected, when solving for the fraction bound (f_{b}), by taking into account the change in intensity of the bound fluorescent ligand, termed g, namely,

$$f_{\text{b}}=\frac{(3-P_{\text{b}})(P_{\text{obs}}-P_{\text{f}})}{(3-P_{\text{obs}})(P_{\text{b}}-P_{\text{f}})+(g-1)(3-P_{\text{f}})(P_{\text{b}}-P_{\text{obs}})} \tag{9}$$

$$f_{\text{b}}=\frac{(r_{\text{obs}}-r_{\text{f}})}{(r_{\text{b}}-r_{\text{f}})+(g-1)(r_{\text{b}}-r_{\text{obs}})} \tag{10}$$

where P_{obs} is the observed polarization, P_{f} is the polarization of the free ligand, and P_{b} is the polarization of the bound ligand. An expression for anisotropy, which takes into account changes in intensity (g), is also shown above where r_{obs} is the observed anisotropy, r_{f} is the anisotropy of the free ligand, and r_{b} of the bound one.

The dissociation constant corresponding to a reversible equilibrium between a protein (P), a ligand (L), and a protein–ligand complex (PL) is given by:

$$K_d = \frac{[P][L]}{[PL]} \tag{11}$$

Determination of this dissociation constant requires determination of the concentration of bound ligand (PL) as a function of the total protein (P_T) and ligand (L_T) concentrations:

$$[PL] = \frac{(K_d + L_T + P_T) - \sqrt{\left[(K_d + L_T + P_T)^2 - 4P_T L_T\right]}}{2} \tag{12}$$

Dissociation constants for the protein–ligand system can then be determined by fitting a plot of f_b vs. concentration to the following equation:

$$f_b = \frac{[\text{Protein}]}{[\text{Protein}] + K_d} \tag{13}$$

2 Materials

All solutions were made up using ultrapure water (specific resistance 17.5 MΩ) collected from a Barnstead Nanopure II at 25 °C and, when required, filtered through a 0.22 μm filter from Millipore. Buffer and protein stocks were stored at 4 °C. Stock solutions of furosemide were stored at room temperature:

1. Monomeric HSA (mHSA): ~1 mM stock solution isolated from lyophilized, 99 % agarose gel electrophoresis, HSA from Sigma (St. Louis, MO, USA). *See* Subheading 3.1 for purification procedure. (We note that lyophilized HSA typically contains about 10–15 % of a covalent dimer and higher oligomers due to exchange of disulfide cross-links within the protein population [16].)
2. Furosemide: 850 μM (0.28 g/L) stock solution was made in 0.05 M HEPES, pH 7.0 (Sigma, St. Louis, MO, USA).
3. Sephacryl S300 buffer: 0.05 M HEPES, pH 7.5, and 0.05 M NaCl. Weigh out 11.9 g of HEPES powder and 2.9 g NaCl. Dissolve in ~900 mL. Adjust the pH to 7.5 with 2 M NaOH. Add ~100 mL of water to make 1 L. Filter through a 0.22 μm and store at 4 °C.
4. Sephacryl S300 column: Contained in a Pharmacia XK16 column with ~120 mL of S300 resin. Column stored in S300 buffer or, for long-term storage, in 70 % ethanol.

5. Amicon Ultra centrifugal filters: Ultracel—10 kDa MWCO from Millipore (Bellirica, MA, USA).
6. Phosphate buffer (PB): 0.05 M phosphate buffer, pH 7.0. Dissolve 0.17 g of potassium phosphate, monobasic, (=KH_2PO_4) and 0.22 g of potassium phosphate, diabasic, (=K_2HPO_4) in 50 mL of water. The pH should be near 7; however, small adjustments with 1 N HCl were done to place it exactly at 7.
7. Fluorescent cuvettes: Suprasil quartz, 10 × 4 mm.

3 Methods

Carry out all experiments at room temperature.

3.1 Isolation of Pure Monomeric HSA from a Lyophilized Source

1. Start by equilibrating the Sephacryl S300 column, which is typically stored in 17 % ethanol, with 5 column volumes of S300 buffer. Our column is ~120 mL and was equilibrated by running 600 mL of S300 buffer through.
2. Dissolve ~50 mg of lyophilized HSA into 1 mL of Sephacryl S300 buffer. Run this solution over the S300 column (*see* **Note 1**).
3. Collect the fractions and record the absorbance at 280 nm (the main protein absorbance wavelength). The absorbance values for the fractions collected from our S300, run at 1 mL/min with 600 μL fractions, are shown in Fig. 1a. Run out fractions around the main peak on a native gel, Fig. 1b, to isolate the fractions containing pure mHSA (*see* **Note 2**).

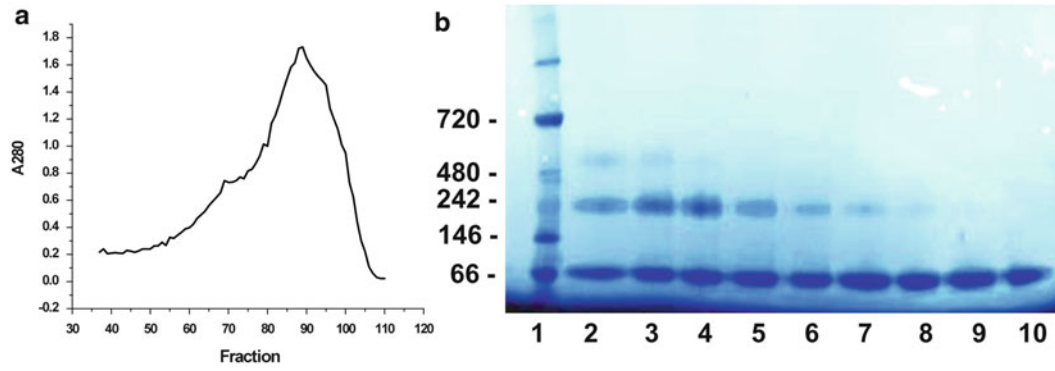

Fig. 1 Isolation of monomeric HSA from a lyophilized source. (**a**) Protein absorbance (A_{280}) of 600 μL fractions collected off a Sephacryl S300 column. (**b**) A native, non-denaturing gel of fractions 59 (*Lane 2*), 65 (*Lane 3*), 71 (*Lane 4*), 77 (*Lane 5*), 80 (*Lane 6*), 84 (*Lane 7*), 89 (*Lane 8*), 94 (*Lane 9*), and 99 (*Lane 10*). Around 3 μg, determined by the A_{280}, of each fraction was loaded. *Lane 1* contained 5 μL of NativeMark (Invitrogen)

4. Pool the fractions containing mHSA and concentrate them in an Ultracel 10 kDa. Once all the fractions have been added and concentrated, exchange the buffer to PB by adding in 4 mL and concentrating the sample. This step needs to be done at least three times. In our case, the stock solution of mHSA was ~1 mM in PB. This monomeric sample is used throughout these experiments.

3.2 Polarization of mHSA During Furosemide Binding

1. Make a 1 μM solution of mHSA in a fluorescence cuvette by diluting a small amount of the concentrated mHSA with PB (near 800 μL). The concentration can be known precisely by measuring the absorbance at 280 nm and using the absorption coefficient of 35,121 M^{-1} cm^{-1} [17].
2. For these experiments, we will be varying the amount of ligand, furosemide, while maintaining a constant concentration of mHSA (*see* **Note 3**). This procedure is done by diluting the excess furosemide with volumes of 1 μM mHSA. To keep things identical throughout the experiment, a 10 mL solution of 1 μM mHSA is made in PB by adding 0.010 mL of the stock mHSA to 9.990 mL of PB.
3. Place the cuvette into the fluorimeter, with polarizers on the excitation and emission side, and measure the polarization of mHSA using 300 nm excitation (*see* **Note 4**). This polarization value is P_f (Eq. 9) and the intensity will be used to calculate g. The emission can be collected either through a monochromator or directly through a filter (*see* **Note 5**).
4. Make a solution containing ~30 μM of furosemide by adding enough ligand from the stock solution to the cuvette and ensure that the final volume is 800 μL (*see* **Note 6**). Measure the polarization and use this value for P_b, assuming all mHSA is bound (Eq. 9). The g value for Eq. 9 can now be determined by dividing the intensity of the bound mHSA (~45,000 counts) by the free mHSA (~420,000 counts). This gives a g value of 0.11.
5. Dilute the sample to 20 μM furosemide by removing 265 μL from the cuvette and adding back 265 μL of the 1 μM solution of mHSA and measure the polarization. Repeat this step to 15, 10, 7.5, 5, 2.5, 1.25, 0.625, 0.530, 0.430, and 0.215 μM.
6. Figure 2a shows the plot of polarization vs. total ligand concentration (M), on a log scale.
7. Using the polarization values and the g value from above, the fraction of bound ligand at each concentration can be determined from Eq. 9. The plot of f_b vs. total ligand concentration (M) is shown in Fig. 2b, along with the best fit using Eq. 13. This fit gives a K_d of 0.46 ± 0.03 μM for furosemide binding to mHSA (*see* **Note 7**).

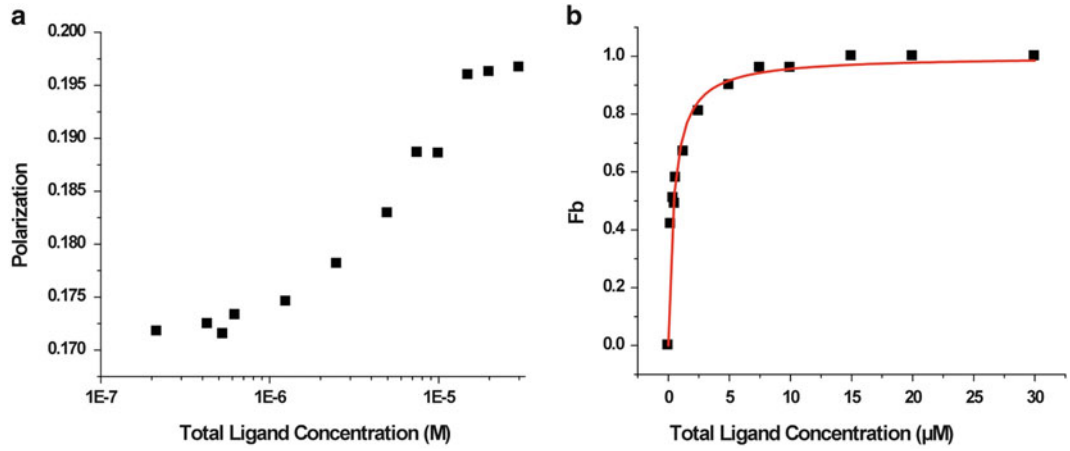

Fig. 2 Steady-state polarization of the intrinsic tryptophan of mHSA during furosemide binding. (**a**) Polarization values of mHSA at varying concentrations of furosemide, plotted on a logarithmic scale, while maintaining 1 μM mHSA. (**b**) Plot of fraction bound furosemide, corrected for changes in intensity, at each concentration of furosemide. Line is the best-fit binding isotherm, Eq. 13, with a K_d of 0.4 μM

3.3 Monitoring the Polarization Change of a Fluorescent Ligand for Determination of the K_d Between Protein–Ligand Interactions

1. In this example, which has been published previously [10], the change in polarization of the fluorescent ligand, mant-GTPγS, binding to a large protein, dynamin 2 (Dyn2), is followed.
2. A stock solution of mant-GTPγS (a fluorescent, slowly hydrolyzable GTP analog) was made in 20 mM HEPES, pH 7.4, 300 mM NaCl, 5 mM $MgCl_2$, 1 mM EDTA, 0.2 mM PMSF, and 0.5 mM DTT [18], and the concentration was adjusted to 1 μM by measuring the absorbance at 354 nm and using the absorption coefficient of 5,700 M^{-1} cm^{-1} [19].
3. The anisotropy of the 1 μM solution was determined by excitation at 354 nm and measuring the polarized emission through a 430 nm long-pass filter.
4. Anisotropy measurements were initially made with 1 μM mant-GTPγS in solution with the GTP-binding protein Dyn2 at 38 μM. The Dyn2 concentration was lowered to ~35 μM by removing a fraction of the sample in the cuvette and adding back an identical volume of 1 μM mant-GTPγS. This step was repeated until the concentration of Dyn2 was 2.5 μM.
5. The plot of anisotropy vs. protein concentration (μM) is shown in Fig. 3 along with the best fit to the anisotropy function (Eq. 11).

3.4 Protein Dimer–Monomer Dissociation Constant Determined by Steady-State Polarization

1. One can also study the strength of association of a protein that forms higher-order oligomers in vitro by polarization. In this case, the ligand bound is the unlabeled protein.
2. We will describe the procedure outlined by Ross et al. [11] who investigated the monomer–dimer equilibrium of endophilin.

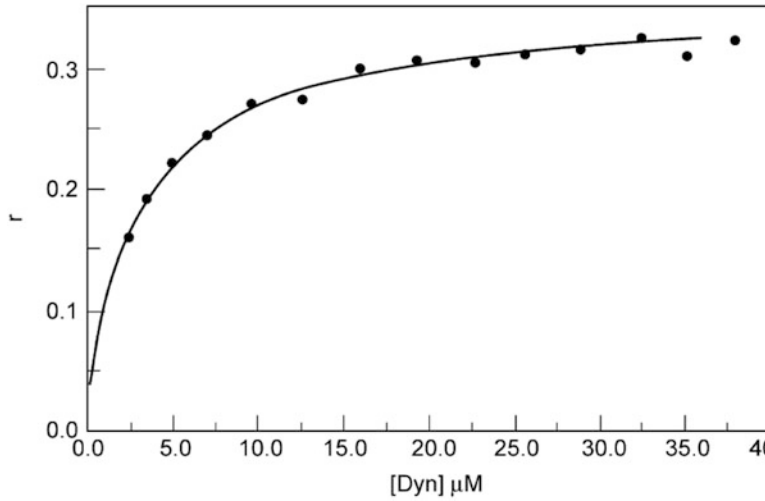

Fig. 3 In this experiment, 1 μM mant-GTPγS (a fluorescent, non-hydrolyzable GTP analog) was present, and the concentration of the GTP-binding protein, Dyn2, was varied by starting at high concentrations followed by dilution. The binding curve was fit to the anisotropy equation (in this case, the yield of the fluorophore increased about twofold upon binding). A K_d of 8.3 μM was found

3. 500 μL of endophilin A2 (4 μM) was reacted with 10 μL of 6 mM Alexa Fluor 488 succinimidyl ester (*see* Fig. 4). The reaction took place in 20 mM HEPES, pH 7.5 with 100 mM NaCl, and was allowed to proceed overnight at 4 °C. The solution was dialyzed (6–8,000 MWCO) against the reaction buffer, and labeling was determined to be 2:1, protein to fluorophore (*see* **Note 8**).
4. The labeled protein was seeded with unlabeled protein to achieve a starting concentration of 20 μM. Anisotropy was recorded by excitation with 488 nm light with the polarized emission being collected directly through a 525 nm long-pass filter (no. 3484; Pyrex) (*see* **Note 9**).
5. The sample was sequentially diluted, with 20 mM HEPES, pH 7.5, and 100 mM NaCl, down to 100 nM protein concentration.
6. Figure 3 shows the polarization as a function of protein concentration and fit to the dimer–monomer binding isotherm with a K_d of 15 μM.

4 Notes

1. The Sephacryl S300 resin is not optimal for separation of 132 and 66 kDa (dimer vs. monomer HSA). Therefore, there will be some overlap of the higher-order oligomers (trimer and dimer)

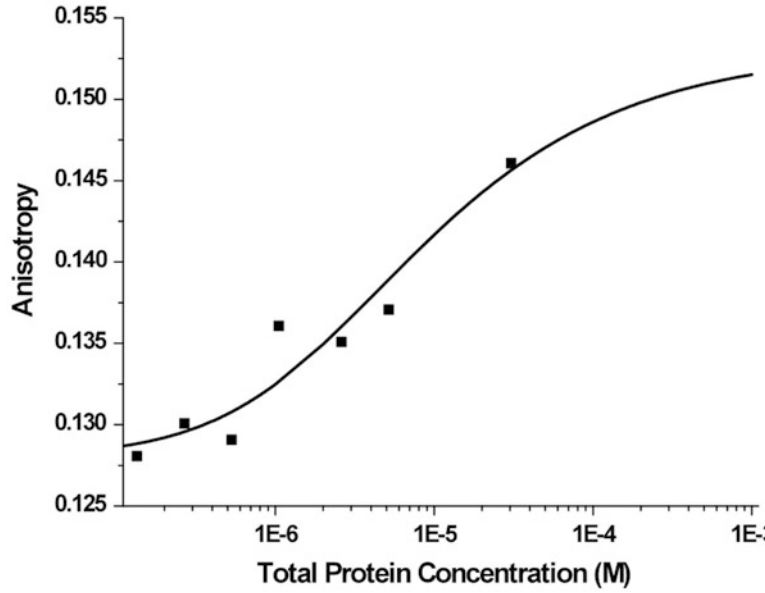

Fig. 4 Steady-state anisotropy of extrinsically labeled endophilin. Anisotropy values, along with the best fit to the binding isotherm, of varying concentrations of Alexa Fluor 488 labeled endophilin. This figure is a modification from a previous publication [10]

with the monomer, which is clearly visible from Fig. 1. To minimize this, run the S300 column at 1 mL/min (or slower) and collect 500 μL fractions. Better separation can be accomplished by using a resin with higher separation in this area, such as an S200 or lower.

2. Lyophilization of HSA causes oligomerization through disulfide linkage exchange [16]. Therefore, a native, non-denaturing gel is needed to see the higher-order oligomers. Fraction 89 (highest point on the main peak) still contains a faint dimer band, and, as such, fractions 90–110 were pooled and concentrated.
3. An issue arises with this ligand as furosemide has an absorption band starting from ~310 nm and fluorescence emission from 350 to 500 nm [20]. The polarized emission from mHSA can be isolated by collecting the emission through a monochromator set at 330 nm or through a band-pass filter centered in the 330 nm region. It is absolutely critical to avoid the emission from furosemide because, without directly exciting furosemide, the emission will result from energy transfer between tryptophan and furosemide. This transfer will cause a decrease in polarization, when in actuality the polarization should increase. And, while it is possible to collect the data through the monochromator, it is highly recommended that an interference filter

be used. This point is stressed as the emission of mHSA is quenched ~90 % at the start of the experiment and more counts results in improved precision of the measurement [21].

4. At this wavelength, we are only exciting tryptophan residues, thus avoiding any potential tyrosine–tryptophan energy transfer, which could lower the observed polarization. We recommend using a 300 nm narrow band-pass filter (ours is FF01-295/15-25 from Semrock) after the excitation monochromator to ensure no excitation light above 305 nm impinges on the sample as the ligand, furosemide, can be excited by wavelengths above 310 nm [20].
5. There are advantages and disadvantages to measuring polarization through a monochromator vs. a filter (long-pass or band-pass). The major advantage is the ability to select a specific emission wavelength. However, the major disadvantage is the dramatic loss in intensity. Another factor that needs to be considered critically when using a monochromator is the *G*-factor, which takes into account the dependency of the transmission efficiency of the monochromator on the polarization direction of the light. This value should be measured for each individual system; however, in the case of directly measuring polarization through a filter, the value is typically around 1 (in our system it is 1.03 ± 0.02). Calculation of this value requires emission intensity collected through excitation and emission polarizers set at horizontal and horizontal (I_{HH}) and horizontal and vertical (I_{HV}):

$$G = \frac{I_{HV}}{I_{HH}}$$

which can then be added to modify Eqs. 1 and 2 to:

$$P = \frac{(I_{\parallel} - GI_{\perp})}{(I_{\parallel} + GI_{\perp})}$$

$$r = \frac{(I_{\parallel} - GI_{\perp})}{(I_{\parallel} + 2GI_{\perp})}$$

The *G*-factor, when measuring through the monochromator at 300 nm, in our system is 2.28 ± 0.03.

6. Ensure that the stock solution of furosemide is concentrated such that volume added is low, minimizing the dilution effect on the mHSA fluorescence. The final volume of the sample is critical because the meniscus needs to be out of the path of the excitation light and the observation volume.
7. Voelker et al. [4] did a study of furosemide binding to HSA using the fluorescence quenching upon binding. Their reported K_d, under similar conditions as our study, was

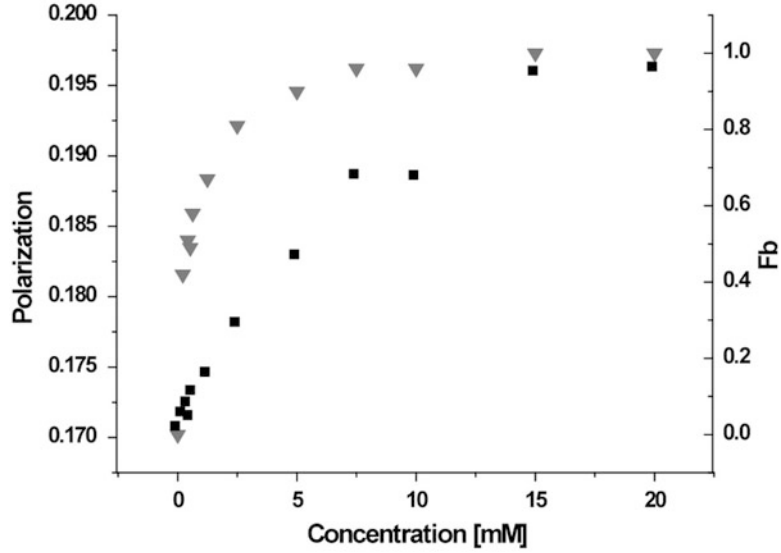

Fig. 5 Corrected and uncorrected f_b of furosemide to mHSA. If the f_b were not corrected for the large difference between bound and free forms from Subheading 3.2, the data would look like the *black squares* with a K_d of ~3.5 μM

0.65 ± 0.05 μM, which is almost identical to the value being reported here. With regard to the intensity change, if we did not take this change into account when calculating f_b (Eq. 9), the plot of f_b vs. total ligand concentration (M) would look like Fig. 5. The gray squares are the corrected f_b values. A K_d of 3.5 μM is obtained when the uncorrected values are fit to Eq. 13. This value is nearly ten times higher than the corrected K_d, 0.4 μM, demonstrating the need for correction.

8. The optimal conditions for labeling a protein must be worked out for each individual case, i.e., what probe concentration, what protein concentration, what pH, how long, and what temperature. After the labeled protein is isolated from the unreactive probe (either by gel-filtration chromatography, spin columns, or dialysis), the extent of labeling must be determined. Clearly, if one knows the extinction coefficient of the protein and the probe at 280 nm and if one knows the extinction coefficient of the probe at a higher wavelength, where the protein does not absorb light, then the labeling ration calculation is straightforward. However, one may not readily know the contribution of the probe at 280 nm. In this case, one should utilize a classic protein determination assay, such as the Bradford method, to determine the protein concentration. If one knows the probe's extinction coefficient at 280 nm, then the proteins coefficient may be estimated from its amino acid sequence using the formula worked out empirically by Pace et al. [17], namely,

$$\varepsilon_{280\text{nm}}(\text{M}^{-1}\text{cm}^{-1}) = (\#\text{Trp})\,(5{,}500) + (\#\text{Tyr})\,(1{,}490) + (\#\text{Cystines})\,(125)$$

9. The Raman peak will be located at 585 nm with 488 nm excitation light. As such, for these experiments, the Raman peak will begin to show at lower and lower intensities due to the long-pass filter allowing all light above 525 nm. Because Ross et al. [11] were not able to maintain a constant intensity, this experiment was only able to go down to ~100 nM (due to Raman contamination). A more complete binding isotherm can be obtained with a higher labeling ratio.

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