APPLICATION NOTE

LIGAND BINDING ASSAYS ON THE BASIS OF FLUORESCENCE ANISTROPY

AN_P22 v.2; 23 Nov. 15, Tim Rasmussen

INTRODUCTION

Fluorescence anisotropy or polarisation provides a sensitive tool to measure the binding of ligands to proteins when a fluorophore is attached to the ligand. This method is particularly useful if no changes in other fluorescence properties are seen. Changes in the anisotropy are caused by changes of the mobility of the fluorophore. This is the case when a small ligand binds to a macromolecule (e.g., proteins) that moves much slower than the ligand free in solution.

For anisotropy measurements linearly polarised light is used for excitation with a polariser placed between light source and sample and the emission intensity is measured dependent on the polarisation plane by using a second polariser between sample and detector\(^6\). The anisotropy is then obtained as shown in Equation 1 where the first subscript indicates the position of the excitation polariser, the second of the emission polariser and \(G(l_{em})\) is an instrumental correction factor \(G(l_{em})=IHH(l_{em})/IHV(l_{em})\). The polarisation either vertical (V) or horizontal (H):

\[
 r = \frac{G(l_{em})I_{VY}(l_{em})-I_{VV}(l_{em})}{G(l_{em})I_{VY}(l_{em})+2I_{VV}(l_{em})}
\]

The anisotropy is sensitive to the mobility of the fluorophore as it may move between excitation and emission resulting in a changed polarisation plane of the emitted light. Thus anisotropy measurements are especially useful when the ligand is labelled because the ligand is immobilised upon binding.

A soluble construct of the ligand binding domain of Kef from \(S.\ denitrificans\) was purified for binding experiments\(^6\). In the shown experiment a high concentration of 54 \(\mu\)M Kef was used which provided low noise levels, but requires corrections during the analysis for the depletion of the ligands.

In this example, a specific fluorescence probe was synthesised, but general probes are commercially available.

A micro fluorescence cuvette (Hellma, 105.254-QS) with 3x3 mm light paths was used to minimise the required sample volume to 100 \(\mu\)l. The temperature was kept constant at 20°C.

METHODS AND MATERIALS

Polarised emission spectra were recorded in an FLS980 Fluorescence Spectrometer equipped with double excitation and emission monochromators. Calcite polarisers were used in the excitation and emission, while for detection a photomultiplier tube detector (Hamamatsu, R928P) with 0.2 s dwell was used.

The binding of the ligand \(S\)-Octan-3-on-1-yl glutathione (OctSG; adduct of the electrophile 1-octen-3-one and glutathione (DNGSH) (synthesised by Conway \textit{et al}., Oxford). The dansyl group was chosen as it is a small fluorophore which reduces the chance of steric clashes during binding to Kef. It was established that this probe binds to Kef and a dissociation constant of \(K_d=6 \ \mu\)M was determined\(^5\). Twice the Kef concentration (100 \(\mu\)M) of DNGSH was used in the competition experiment.

The binding of the ligand \(S\)-Octan-3-on-1-yl glutathione (OctSG; adduct of the electrophile 1-octen-3-one and glutathione) was used as an example\(^7\) (synthesised by Conway \textit{et al}., Oxford). This ligand, as well as DNGSH, was dissolved in a titration device.

It was a prerequisite to establish the \(K_d\) for the fluorescence probe DNGSH beforehand (see above). In addition, it is required to approximate properties of the bound DNGSH before the competition experiment can be started.
LIGAND BINDING ASSAYS ON THE BASIS OF FLUORESCENCE ANISOTROPY

Therefore, a reverse titration of a small amount DNGSH with increasing concentration of Kef was performed. It was established that the anisotropy of DNGSH bound to Kef is \( r_0 = 0.180 \) and that the fluorescence intensity is 4 times as high as for the free ligand \( (Q=4) \). The anisotropy of the free DNGSH was measured directly \( r_0 = 0.020 \).

DNGSH was added to the Kef sample and the anisotropy was recorded. Then the ligand OctSG was added stepwise, the sample was equilibrated for 5 min, and the anisotropy was recorded.

RESULTS - DISCUSSION

The raw data of the OctSG titration are shown in Figure 1. To simplify analysis, mean values for the anisotropy were calculated over the recorded wavelength range for each titration step. These are shown as data points in Figure 2.

![Figure 1: OctSG titration to Kef in the presence of DNGSH. Anisotropies were recorded in the emission range from 540 nm-560 nm. The excitation wavelength was set to 340 nm for excitation of the dansyl group. The anisotropy was measured over the emission range of 540 nm-560 nm where the dansyl group shows strong fluorescence. Sift widths of \( \Delta \lambda_{ex} = 2 \) nm and \( \Delta \lambda_{em} = 3 \) nm, step=1 nm and tintegr=0.2 s were used.](image1)

![Figure 2: Fit of the dissociation constant for the titration concentrations used in the experiment. The inset shows an overview of the experiment.](image2)

The addition of OctSG solution causes an increase of the total volume and stepwise dilution of Kef and DNGSH. The accurate concentrations were calculated for each step.

Data were analysed for a single site binding model considering the depletion of the probe DNGSH and the ligand OctSG due to binding to Kef. The experimental observed anisotropy \( r_{obs} \) can be expressed as a sum of anisotropies of the free and bound probe, \( r_f \) and \( r_{fML} \), under consideration of their fluorescence intensities, \( I_{ML} \) and \( I_f \):

\[
r_{obs} = \frac{r_{fML}I_{ML} + r_fI_f}{(I_{ML} + I_f)}
\]

Intensities are defined with the fraction of bound ligand \( f = [ML]/[L] \) and the quantum yields as \( \Phi = \Phi_{ML}f + \Phi_f(1-f) \). This can be substituted into Equation 2 and solved for \( f \) using the ratio of quantum yields \( Q = \Phi_{ML}/\Phi_f \):

\[
f = \frac{1}{1 + Q(r_{obs} - r_{ML})/(r_f - r_{obs})}
\]

Equation 3 can be rearranged for use in the fitting together with expressions for \( f \):

\[
r_{obs} = \frac{r_fB - r_f + r_{fML}}{1 + \frac{r_fB}{Q}}
\]

Definitions for the dissociation constants DNGSH and OctSG lead together with mass conservations to a cubic equation of the DNGSH-Kef complex concentration, \([ML]\). One meaningful solution can be obtained and substituted as \( f \) into Equation 4 (for details see suppl. information ref). A MATLAB program was written for fitting of the apparent number of binding sites, \( n \), and the dissociation constant for OctSG, \( K_d(OctSG) \). A dissociation constant of \( K_f = 7.1 \) \( \mu \)M for OctSG was found.

REFERENCES