Fusion Protein Complexes Analysed by CG-MALS - Non-equivalent, Multivalent Interactions

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Quantifying binding affinity between species with multiple binding sites can present a significant challenge to many biophysical characterisation techniques. Composition-gradient multi-angle light scattering (CG-MALS) provides direct measurement of affinity and absolute stoichiometry for a wide variety of interactions, including multivalent interactions, without the need for surface immobilisation or tagging.

A model fusion protein, Y, was engineered with two binding sites for its ligand, X. The interaction was quantified using two CG-MALS experiments. The first experiment quantified the interaction between X and Y at a high-affinity binding site and suggested the presence of a second low-affinity binding site. A simulation based on these results led to the design of a follow-up experiment to quantify the affinity at the weaker binding site and confirm that no additional higher order species were formed.

The Molecular Interactions Research Group (MIRG) of the Association of Biomolecular Resource Facilities (ABRF) developed two proteins to use as a model system for investigating multivalent interactions. In 2012, these proteins were used in a benchmark study to test the capabilities of common molecular interaction technologies. Proteins X and Y were sent to a panel of participants with specific instructions for analysing the interaction via surface plasmon resonance (SPR) to determine the binding affinity and stoichiometry. Instructions were given for immobilising protein Y as the ligand in the SPR assay, and the organisers recommended sampling analyte (protein X) concentrations of 1 nM to 300 µM.

Extensive binding studies had been performed with SPR, isothermal titration calorimetry (ITC), and analytical ultracentrifugation (AUC) to quantify the affinities at each site (Table 1).

Table 1. Summary of measured binding affinities between barstar to bivalent barnase fusion, as measured by different techniques.

<table>
<thead>
<tr>
<th>Technique</th>
<th>Measured Binding Affinity, Kd (µM)</th>
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<tr>
<td>SPR</td>
<td>Binding Site 1: 0.350 ± 0.140; Binding Site 2: 79 ± 45</td>
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<tr>
<td>ITC</td>
<td>0.008 – 0.034; 9 – 17</td>
</tr>
<tr>
<td>AUC</td>
<td>0.005 – 0.040; 16 – 46</td>
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To adapt the SPR experimental guidelines for a CG-MALS experiment, the CALYPSOTM software’s simulation tool was used to identify stock concentrations that would provide quantitation of a wide range of affinities and stoichiometries. Interactions with Kd ranging from 10 nM to 10 µM and between 1 and 4 binding sites were considered. As a result of the simulations, stock concentrations of 4.8 µM protein X and 800 nM protein Y were chosen to provide Kd between 10 nM and 1 µM. The simulation helped determine that analysis of weaker affinities or complex stoichiometries requires a second experiment, where the results of the initial measurement are used to optimise conditions for the second experiment.

Materials and Methods

Reagents and instrumentation

Proteins X and Y were provided by the MIRG Benchmark Study at concentrations of 9 mg/mL and 2.68 mg/mL, respectively. All experiments were performed in acetate buffer (50 mM NH₄CH₃COO, 100 mM NaCl, 0.1% EDTA, 0.01% NaN₃, pH 8.0) filtered to 0.1 µm.

The interaction between proteins X and Y was quantified by composition-gradient multi-angle static light scattering (CG-MALS), automated by the Wyatt Calypso® system. Inline filters of 0.1 µm pore size were installed in the Calypso. Protein and buffer solutions were delivered by the Calypso to a UV/Vis concentration detector (Waters) and DAWN® MALS detector. Protein solutions were prepared at the stock concentrations specified for each experiment, filtered to 0.02 µm, and loaded onto the Calypso. The Calypso prepared different compositions of protein X, protein Y, and buffer, and delivered the mixed solution to the Calypso to a UV/Vis concentration detector (Waters) and DAWN® MALS detector.

Experiment 1

Proteins X and Y were diluted to ~0.05 mg/mL and ~0.02 mg/mL (~0.8 µM and ~4.8 µM), respectively, in running buffer and filtered to 0.02 µm prior to loading on the Calypso. The experiment consisted of three composition gradients: 1) a concentration gradient in protein Y to quantify its molecular weight and any self-interactions, 2) a hetero-association (‘crossover’ gradient) consisting of fourteen compositions of X and Y to identify the

For illustrative purposes only. Binase fusion protein (multi-coloured, pdb1BUJ and pdb2RBI) and two barstar molecules (red, pdb2HXX).
interaction affinity and stoichiometry, and 3) a concentration gradient in protein X to quantify its molecular weight and any self-interactions. After each injection, the flow was stopped for 60 seconds (single-component gradient) or 180 seconds (crossover gradient) to allow the solution to come to equilibrium.

**Experiment 2**

Proteins X and Y were diluted to ~0.1 and ~0.4 mg/mL (~30 and ~3 µM), respectively, in running buffer and filtered to 0.02 µm prior to loading on the Calyxo. The experiment consisted of a single hetero-association (‘crossover’ gradient) consisting of nine compositions of X and Y to identify the interaction affinity and stoichiometry (Figure 1, right). After each injection, the flow was stopped for 180 seconds to allow the solution to come to equilibrium.

The equilibrium light scattering intensity and the fit is less than 1% for the majority of the data (Figure 3, bottom). This broader curvature is typically indicative of higher-order stoichiometries. For much of the hetero-association gradient, the measured light scattering intensity is up to 4% greater than what is allowed by a 1:1 stoichiometry. The CG-MALS data reach a maximum at approximately equimolar concentrations of proteins X and Y.

**Results and Discussion**

Two CG-MALS experiments were performed to determine the affinity and stoichiometry of the multivalent interaction between proteins X and Y. The initial experiment, carried out at low concentration, quantified complex formation at a high-affinity binding site and detected a possible second, lower-affinity binding site on Y for X. Several interaction models that fit the first data set reasonably well, were examined with the CALYPSO software simulation tool, and a follow-up experiment was designed to confirm and quantify the lower-affinity binding site.

**Initial experiment results and analysis**

In Experiment 1, light scattering and concentration data were collected for fourteen compositions of X and Y to quantify their hetero-interaction. At first glance, the light scattering data from the low-concentration CG-MALS experiment appear consistent with a 1:1 stoichiometry. The CG-MALS data reach a maximum at approximately equimolar concentrations of proteins X and Y. Fitting the data with a 1:1 model yields a binding affinity around 10 nM (Figure 3, black dashed line). However, this fit slightly underestimates the measured data for compositions in which X is in excess of Y. For much of the hetero-association gradient, the measured light scattering intensity is up to 4% greater than what is allowed by a 1:1 stoichiometry. The difference is consistent and correlated and does not appear to be random error (Figure 3, bottom). This broader curvature is typically indicative of higher-order stoichiometries.

Including the formation of the X2Y complex in addition to the XY complex better captures the curvature in the measured light scattering data (Figure 3, solid red line). The reported χ² value for the two binding-site model was slightly improved compared to the one binding-site model (0.989 and 1.16, respectively). Furthermore, the error between the measured intensity and the fit is less than 1% for the majority of the data (Figure 3, bottom).

When considering multivalent interactions, the CALYPSO software does not require that the affinity at each binding site be equivalent; rather, the equilibrium association constant for each complex is determined independently. In this experiment, the equilibrium association constants determined by fitting to multivalent models do not support the assumption of equivalent binding sites on Y for X.

**Follow-up experiment results and comparison to other techniques**

With the Experiment 2, the interaction was measured at concentrations > 10 µM X. In addition, the majority of the data were collected under conditions of excess X to favour binding at both possible sites. As shown in Figure 5, considering only a 1:1 interaction clearly does not represent the measured light scattering signal (black dashed line). The best fit (Figure 5, red) requires the formation of XY and X2Y. Fitting the combined data set yielded dissociation constants as follows: Kd,1 = 10 nM and Kd,2 = 14 µM.

The CG-MALS results are in good agreement with the published ITC and AUC analyses for these molecules (Table 1) [3]. Like CG-MALS, both ITC and AUC are solution-based measurements and do not experience artifacts caused by surface interactions, as SPR can. Since light scattering measures molar mass directly, CG-MALS presents additional advantages as a biophysical technique. As shown in Figure 4, the molar mass and self-association parameters for each binding partner can be assessed to understand the oligomeric state of the starting material. Furthermore, the measured molar mass provides a clear readout of the interaction, as compared to a secondary response, such as a fluorescent signal, ‘resonance units’ or heat of reaction. Finally, CG-MALS experiments can be performed relatively quickly to achieve binding affinity and stoichiometry. Each experiment was complete within 1-2 h (Figure 1), whereas similar quantification by AUC required 18-24 h [3].
Quick and Easy and Spectroscopy Sample Preparation

The Fritsch Vibrating Cup Mill PULVERISETTE 9 offers many practical advantages in all areas in which hard, brittle and fibrous material must be ground extremely quick down to analytical fineness. The instrument is indispensable for fast sample preparation, for example in spectroscopy preparation, ore and geology laboratories, mining and metallurgy, ceramics industry, agriculture and environment, infrared and X-ray fluorescence analysis.

The PULVERISETTE 9 is very powerful, especially quiet, simple to operate, quick to clean, has a well-conceived drive concept and the grinding set is safe and easy to tension. The instrument is perfect for loss-free grinding results in extremely short grinding time due to up to 1500 rpm. Grinding sets in 5 different materials and 3 different sizes from 50 ml to 250 ml volume are available.

No similar mill offers a more convenient operation: The working position is ergonomically optimised, the ease of cleaning is without match. The grinding sets are especially light and are equipped with heat insulated handles, and do not have to be placed directly on the vibrating plate in the centre of the mill. The grinding set is simply placed on the guide rail, an anti-rotation lock enables easy movement to the final position and the safety switch checks the firm position. This protects your back and saves energy and time. The grinding set is tensioned in seconds using a well-thought out one-hand lever.

The setting of the grinding time precisely the second, the pause periods and rotational speed as well as the programming and storage of grinding cycles is done via the guide rail, an anti-rotation lock enables easy movement to the final position and the safety switch checks the firm position. This protects your back and saves energy and time.

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Conclusions

CG-MALS, automated by the Calypso, provides rapid, reliable quantification of interaction affinity and stoichiometry. Often, researchers have some knowledge of the biology or presumed interaction between two species. However, even when that is not the case, the CALYPSO software simulation tool can provide ideal conditions to begin characterising the interaction. Further refinement based on initial results can provide conditions for complete characterisation of complex multivalent interactions.

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References


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