Probing the Interactions Among Three Proteins to Evaluate Whether an Antibody and Inhibitor Compete for the Same Binding Site on an Enzyme

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Multi-Angle static Light Scattering (MALS) is a powerful tool for quantifying multiple types of protein-protein interactions. This article provides an account of research conducted to study the interactions between three proteins: human thrombin–α (Thr), antithrombin III (AT), and an anti-thrombin monoclonal antibody (Ab) to evaluate whether the antibody can recognise AT-inactivated Thr.

Introduction

Composition-gradient multi-angle light scattering (CG-MALS) and dynamic light scattering (CG-DLS) quantify the affinity and stoichiometry of equilibrium protein-protein interactions in solution, as well as nonspecific interactions leading to thermodynamic nonideality. In previous studies, CG-MALS was applied to characterise the reversible equilibrium between human thrombin–α and an anti-thrombin antibody, revealing the expected 2:1 stoichiometry and equilibrium dissociation constant K_D = 8.8 nM [1]. In addition, the second order association rate constant for the covalent association of human thrombin–α to antithrombin was calculated using time-dependent MALS TD-MALS [2].. In this article, the CG-MALS is extended to probe the recognition of the bound thrombin-antithrombin complex by the same antibody.

Experimental

Reagents and Instrumentation

For each experiment, human antithrombin III (AT), human thrombin–α (Thr) and mouse monoclonal anti-human antibody (Ab) were diluted to the appropriate concentrations in phosphate buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.76 mM KH_2PO_4, pH 7.4). The CG-MALS experiments were performed with a Calypso II composition gradient system (Wyatt Technology Corporation, Santa Barbara, CA), which prepared different compositions of protein and buffer and delivered them to an online UV/Vis detector (Waters Corporation, Milford, MA) and DAWN HELEOS MALS detector (Wyatt). Inline filter membranes with 0.1µm pore size were installed using a Calypso for sample and buffer filtration. Control of the Calypso pumps, data acquisition, and analysis were performed with the Calypso software.

Size exclusion chromatography (SEC) was performed using a column with 300 Å pore size (030S5 column, Wyatt), with injections performed by an HPLC pump and autosampler (Agilent Technologies, Santa Clara, CA). The separation between the covalent Thr-AT complex and Thr and AT monomers was confirmed by SEC coupled with multi-angle light scattering (SEC-MALS) using a UV detector, DAWN HELEOS, and Optilab rEX refractive index detector (Wyatt).

Determination of Three-Component Binding

For initial determination of Ab binding to bound Thr-AT complex, CG-MALS experiments were performed such that Ab solution was exposed to an unfractionated mixture of Thr, AT and Thr-AT complex, as follows. Thr and AT were diluted to stock concentrations of 24µg/ml and 80 µg/ml, respectively, in PBS, and each solution was filtered to 0.02µm. Equal volumes of filtered stock solution were combined and allowed to incubate at room temperature for ~2 hours with gentle shaking. Ab was diluted to 22µg/mL in PBS and filtered to 0.02µm. A composition gradient was performed, consisting of six compositions of premixed AT + Thr solution at constant concentration of 25µg/mL and Ab concentrations from 0 to 8 µg/mL. For each composition, the Calypso mixed the appropriate volumes of AT-Thr solution and Ab and delivered the solution to the downstream detectors. After each injection, the flow was stopped to allow any binding interactions to come to equilibrium. The measured weight-average molar mass of each composition at equilibrium was used to determine qualitatively whether the antibody would recognise the bound Thr-AT complex.

To quantify the affinity of the Ab-complex binding, a second composition gradient was performed with multiple compositions of Ab and purified Thr-AT complex *(Figure 1)*. Thr and AT were diluted to 2mg/mL each in PBS, mixed at a 1:1 ratio, and allowed to incubate at room temperature 0.5-1 h. Pre-mixed aliquots of 0.1mL were injected onto an SEC column, and fractions of bound Thr-AT complex were collected at the outlet of the UV detector. Multiple injections were performed and the fractions pooled and filtered to 0.1µm, with a final protein concentration ~70µg/mL. The purified Thr-AT complex was then used in further CG-MALS experiments. Ab was diluted to 15µg/ml in PBS and filtered to 0.02µm. The interaction between purified Thr-AT complex and Ab was measured using a single "crossover" gradient consisting of eight compositions of Ab and Thr-AT complex. For each composition, the Calypso mixed purified Thr-AT complex with Ab, injected the solution into the UV and MALS detectors, and stopped the flow to allow all binding reactions to come to equilibrium. The light scattering data as a function of composition was fit to the appropriate binding model to quantify the interaction stoichiometry and equilibrium dissociation constant, K_D, at each binding site [3].

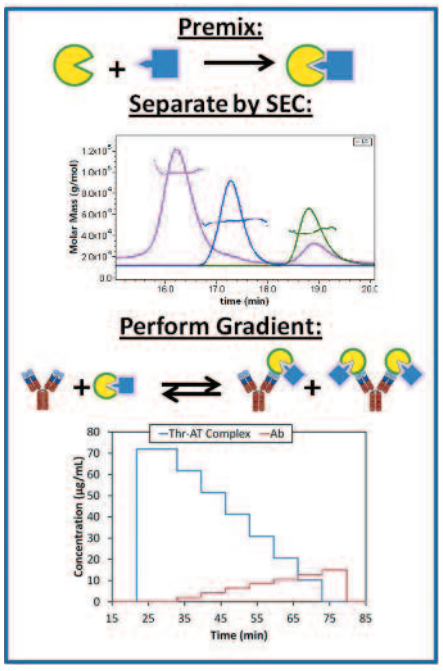


Figure 1. Strategy for quantifying the binding between the covalent thrombin-antithrombin complex and an anti-thrombin antibody

Results

Interaction of Ab with Unfractionated Thr-AT Complex

Once Thr was bound irreversibly to AT, the ability of Ab to retain the Thr-binding activity was measured with CG-MALS.

In a preliminary experiment, AT and Thr were pre-mixed and the reaction allowed to come to completion before loading the unfractionated solution on the Calypso and performing a crossover gradient with this complex solution and Ab. Although binding was evident.

The weight-average molar mass measured by light scattering was significantly less than expected for an interaction between Ab and Thr-AT complex with 2:1 stoichiometry and K_D ~9 nM per binding site, the affinity previously measured for Ab binding pure Thr [1] *(Figure 2)*.

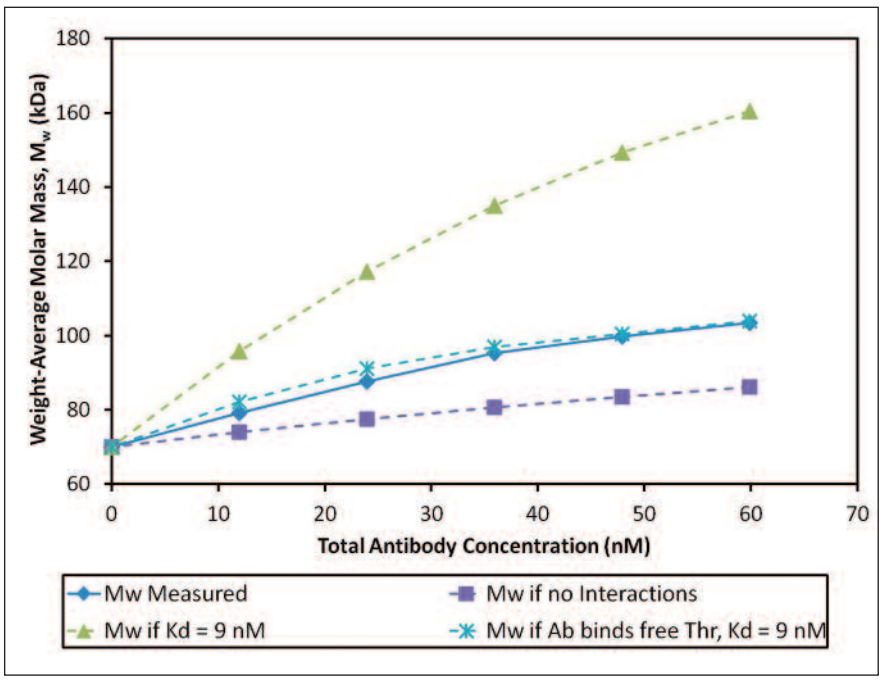


Figure 2. The equilibrium M_W for a composition gradient with constant concentration of unfractionated Thr, AT, and Thr-AT complex (25 µg/mL total protein) and varying concentrations of Ab indicated binding was occurring but could not differentiate between binding with decreased affinity and Ab binding only free Thr in solution.

The decreased Ab-binding could be explained by two possible mechanisms: 1) the Ab bound the Thr-AT complex with decreased affinity compared to free Thr or 2) the Ab did not bind Thr-AT complex, and the observed increase in M_W resulted from Ab binding free Thr in solution.

Previous experiments had shown that ~23% of the Thr was incompetent to binding AT [2]; thus, the observed binding could have resulted from Ab interacting with this incompetent Thr left in solution rather than the Thr-AT complex. Under the experiment conditions, it was impossible to tell from which mechanism the measured M_W resulted.

Interaction of Ab with Purified Thr-AT Complex

In order to distinguish between the two possible mechanisms, bound Thr-AT complex was separated from unbound Thr and AT via SEC *(Figure 3)*. The fraction of Thr that is incompetent for binding to AT is evident as a secondary peak in the SEC chromatogram for the pre-mixed solution. After collecting the appropriate peak, an aliquot was re-applied to the column to confirm that no dissociation had occurred and there was negligible contamination by free Thr.

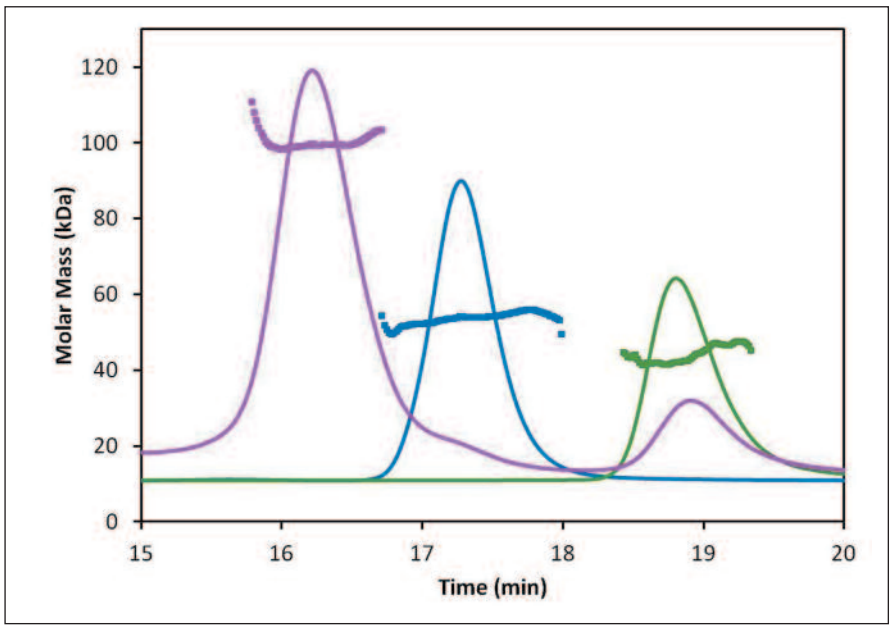


Figure 3. Overlay of size exclusion chromatograms for pure Thr (green), pure AT (blue), and a mixed solution of Thr and AT (magenta). Molecular weights measured by MALS correspond to the expected values. Fractions of covalently bound Thr-AT complex were collected for CG-MALS analysis.

A CG-MALS experiment, consisting of a single crossover gradient with Ab and purified Thr-AT complex, revealed the antibody bound the Thr-AT complex with the expected 1:2 stoichiometry *(Figures 4 & 5)*. Since AT was covalently bound to the Thr active site, this potential binding site was inaccessible to the Ab, indicating that the antibody recognises an epitope on thrombin other than the active site. Previous binding assays also support this assertion. The analysis of Thr-Ab binding indicates no incompetent Thr, suggesting the binding epitope is always accessible. On the other hand, the binding of AT to Thr, which requires the active site to be accessible and/or properly folded, indicates that ~23% of Thr presented an active site that was incompetent for binding AT [2]. Taken together, these data confirm that the antibody binds thrombin away from the active site.

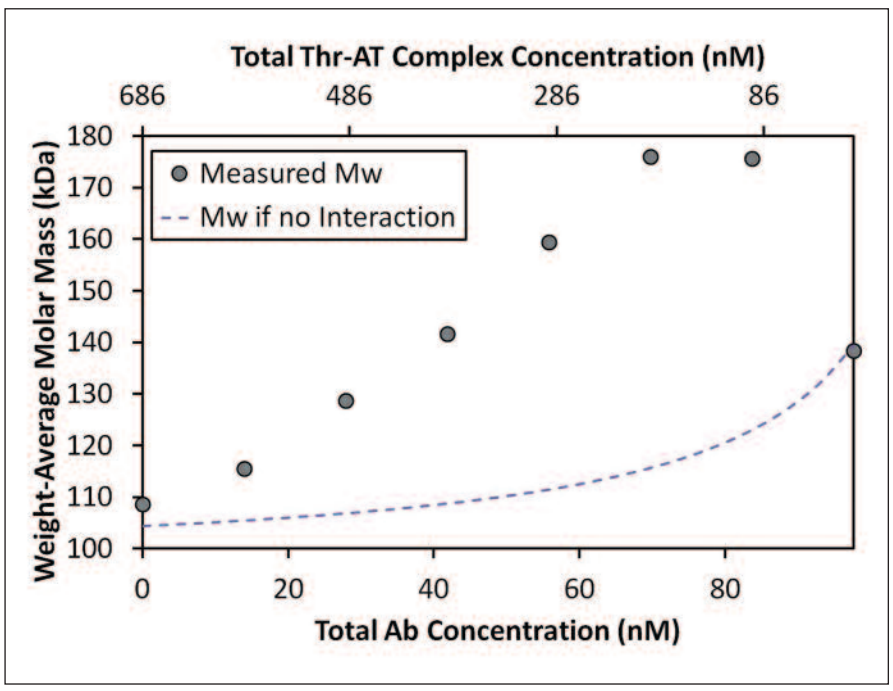


Figure 4. Measured M_W by MALS for composition gradient with varying concentrations of purified Thr-AT complex (10-70µg/mL) and Ab (1-10 µg/mL), indicating an interaction between the Ab and Thr-AT complex. The M_W measured by light scattering increases as the antibody binds the Thr-AT complex. The maximum molecular weight occurs at an overall molar ratio of 1 Ab: 2 Thr-AT complex, indicating a 1:2 stoichiometry.

Although binding of the antibody to the Thr-AT complex is evident, the measured affinity of the antibody for the Thr-AT complex is ~28x decreased as compared to the affinity for pure Thr, with K_D = 250 nM. This may indicate that AT imparts some steric hindrance for antibody-binding. Alternatively, the binding of Thr to AT may lock the enzyme in an inactive conformation [4] which has lower affinity for the antibody than the native or allosterically activated Thr.

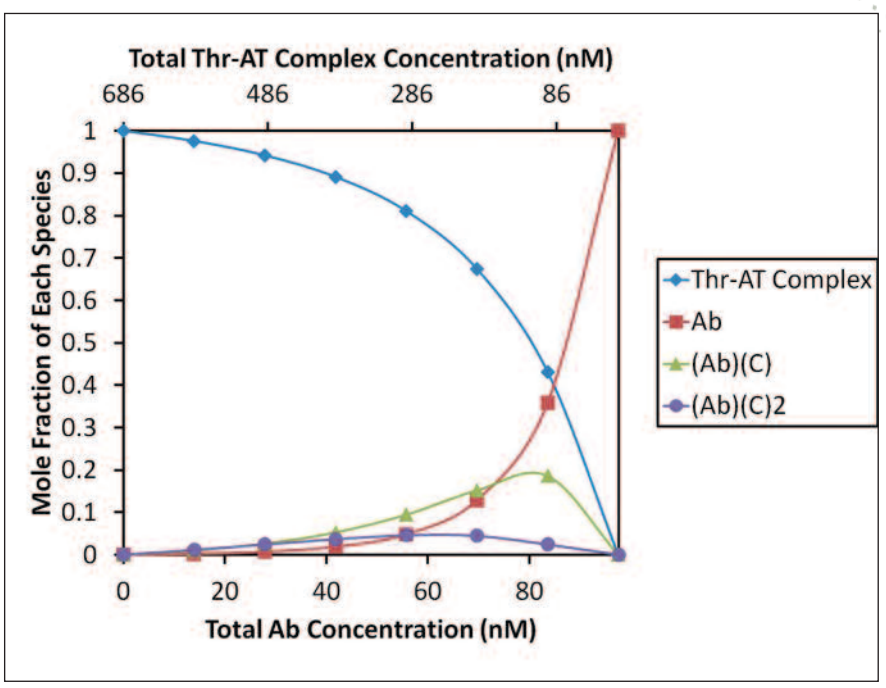


Figure 5. The best fit of LS and concentration data for crossover gradient with purified Thr-AT complex and Ab indicates 1:2 (Ab):(Thr-AT complex) stoichiometry with decreased affinity compared to Thr:Ab alone. Based on these data, the molar composition of each species can be calculated.

Conclusion

MALS is unique among biophysical characterisation techniques because it enables the simultaneous quantification of multiple species present in solution. SEC-MALS confirmed the irreversible binding of AT and Thr and enabled unequivocal separation of the purified complex for further characterisation. CG-MALS quantified the affinity and stoichiometry of the multivalent, equilibrium interaction between Ab and the Thr-AT complex, confirming the binding of the antibody to AT-inactivated thrombin but with decreased affinity. Thus, probing macromolecular interactions by light scattering provides a robust, complementary biophysical technique for understanding complex protein-interaction networks.

References

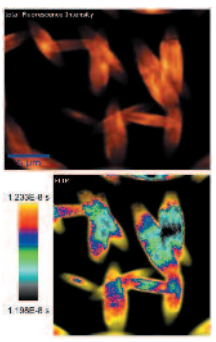
(1) "Measuring the Interaction Between Thrombin-α and an Anti-Thrombin Antibody." Wyatt Technology Corp. Published 7 Jan 2013. www.wyatt.com/files/literature/Calypso_antithrombinantibody.pdf

(2) "Determining the Kinetics of Covalent Thrombin-Antithrombin Association." Wyatt Technology Corp. Published 7 Jan 2013. www.wyatt.com/files/literature/Calypso_covalentantithrombin.pdf

(3) Some, D. and Kenrick, S. (2012) "Characterization of Protein-Protein Interactions via Static and Dynamic Light Scattering. In Protein Interactions, Cai, J. And Wang, R.E. (Eds.), InTech, DOI: 10.5772/37240. Available at: www.intechopen.com/books/protein-interactions/characterization-of-protein-protein-interactions-via-static-and-dynamiclight-scattering

(4) Gandhi, P.S., et al. Proc. Natl. Acad. Sci. 2008 105, 1832.

Time-Resolved Single Photon Counting Measurement Option Introduced



At Pittcon 2013 **WITec** launched StrobeLock, a time-correlated single photon counting measurement option. The imaging modes include Fluorescence Lifetime Imaging and Time-resolved Luminescence Microscopy, which can be integrated with the WITec alpha300 and alpha500 microscope series.

StrobeLock facilitates the acquisition of additional material contrasts hidden in the time function of a fluorescence or luminescence signal and allows them to be perfectly linked with Raman, SNOM or AFM imaging. It enables a variety of measurement possibilities for an improved and more comprehensive understanding of a sample's properties and is specifically suited for materials science.

The modular design of the WITec microscopes facilitates user-friendly combination of the StrobeLock module with the WITec confocal microscope series. StrobeLock is comprised of a pulsed excitation laser system combined with a Time-Correlated Single-Photon Counting (TCSPC) detector. The possibility to switch between time-resolved and conventional mode enables the microscope user to conveniently choose the preferred measurement technique.

"StrobeLock complements the modular WITec microscope systems with customised solutions for the most accurate Fluorescence Lifetime Imaging and Time-Resolved Luminescence Microscopy." said Dr Joachim Koenen, WITec co-founder and Managing Director "This exceptional development significantly extends the capabilities of the WITec microscope series and opens a new field of application for a more comprehensive sample characterisation."

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