Electronic Supplementary Material (ESI) for Analyst.

Pre-Equlibration Kinetic Size-Exclusion Chromatography with Mass Spectrometry Detection (peKSEC-MS) for Label-Free Solution-Based Kinetic Analysis of Protein-Small Molecule Interactions

Jiayin Bao,[a] Svetlana M. Krylova,[a] Leonid T. Cherney,[a] J.C. Yves Le Blanc,[b] Patrick Pribil,[b] Philip E. Johnson,[a] Derek J. Wilson[a] and Sergey N. Krylov[a]*

[a] *Department of Chemistry and Centre for Research on Biomolecular Interactions, York University, Toronto, Ontario M3J 1P3, Canada*
[b] *AB Sciex, 71 Four Valley Dr, Vaughan, Ontario L4K 4V8, Canada*
E-mail: skrylov@yorku.ca; Fax: +416-736-5936; Tel: +416-736-2100 x 22345

Experimental Details

Chemicals and materials. Methotrexate (MTX) was purchased from Sigma-Aldrich (Oakville, ON, Canada). The *E. coli* Rossetta-gami™ 2(DE3) competent cells was purchased from EMD Millipore (PA, USA). Sepharose Fast Flow column, Phenyl Sepharose 6 Fast Flow column and Sephacryl S-100 size exclusion column were purchased from GE Healthcare (Toronto, ON, Canada). The 30,000 MW cutoff Amicon® Ultra 15 mL centrifugal filter devices were purchased from EMD Millipore (Nepan, ON, Canada). All other reagents were purchased from Sigma-Aldrich (Oakville, ON, Canada). All solutions were made using deionised water filtered through a 0.22 μm filter (Millipore, Nepan, ON, Canada).

Protein expression and purification. The plasmid pFW117.1 that contains folA (*E. coli* DHFR) gene was transformed into the competent *E. coli* Rossetta-gami™ 2 (DE3) cells. 1L bacterial culture was growing at 37°C, 260 rpm shaking until the OD_{600} reached 0.8. The culture was then induced with 1.0 mM IPTG and continues growing at 20°C, with 260 rpm shaking overnight. After induction, the cells were collected by centrifugation at 5500 rpm for 1 hour at 4°C. The cell pellet was resuspended in 20 mM Tris-HCl pH 6.2, 0.5 mM EDTA, 0.5 mM DTT , 0.02 % N_aN_3 w/v (buffer A), and then sonicated on ice with 15 s on and 55 s off intervals for 4 min at 60 % amplitude. The lysate was centrifuged at 5500 rpm for 1 hour at 4°C and the supernatant containing DHFR was loaded onto the Q Sepharose Fast Flow column previously equilibrated with buffer A. The DHFR elution was achieved by using a NaCl gradient from 0 to 1.0 M in buffer A, and DHFR eluted at concentrations of 0.4 M NaCl. The fractions containing DHFR were concentrated and dialyzed against 50 mM sodium phosphate pH 7.0 and 1.0 M $(NH_4)_2SO_4$, 0.02 % N_aN_3 w/v (buffer B). The DHFR containing solution was then loaded onto the Phenyl Sepharose 6 Fast Flow column, which was pre-equilibrated with buffer B. The DHFR elution was achieved using 1.0 to 0 M $(NH_4)_2SO_4$ gradient in buffer B, and DHFR eluted at 0.5 M $(NH_4)_2SO_4$. The fractions containing DHFR were concentrated and dialyzed with 100 mM sodium phosphate pH 7.0, 150 mM NaCl, 0.5 mM EDTA, 0.02 % N_aN_3 w/v (buffer C), and then loaded onto a Sephacryl S-100 size exclusion column. All buffer flow rates were 1 mL/min on an

ÄKTA-FPLC system. The purified DHFR was dialyzed against 30 mM ammonium formate pH 7.2 (buffer D).

Instrumentation. The Allegra 21R centrifuge with S4180 rotor was purchased from Beckman Coulter (ON, Canada). ÄKTA-FPLC system was purchased from GE Healthcare (Toronto, ON, Canada). The Shimadzu UFLCXR with Agilent Bio SEC-3 was used for all experiments. Bio SEC-3 Size exclusion chromatography (SEC) column was purchased from Agilent (Mississauga, ON, Canada). The column has 3 µm particle size, 150 Å pore size, 4.6 mm inner diameter and 300 mm length. The AB Sciex QTRAP 6500 with IonDrive Turbo V Source (Concord, ON, Canada) was used for small molecule detection and quantification. Isothermal titration calorimetry (ITC) experiments were performed by using a MicroCal iTC200 system (Northampton, MA, USA).

Pre-equilibration KSEC with MS detection. Buffer D was used for all sample preparation and separation. The pre-equilibration binding mixture was made by incubating 20 nM MTX with different concentrations of DHFR (20 nM – 80 nM) for 10 min at 20°C. 10 µl of binding mixture was injected in to HPLC and separation was conducted at a flow rate of 0.3 mL/min 20°C. Atmospheric-pressure chemical ionization (APCI) was used to ionize MTX with positive ion mode. The source temperature (TEM) was 300°C, the ionization energy (IS) was 5,500 V, and the de-clustering potential (DP) was 125 V. The MRM mode was used to select the ion of 445.2/308.2 (Q1/Q3) by using the collision energy (CE) at of 28 V. All binding experiments were done in triplicates. Fitting the experimental peKSEC-MS chromatograms with the simulated ones was carried out by using COMSOL Multiphysics 4.3a commercial software (COMSOL Group, Palo Alto, CA).

Isothermal titration calorimetry analysis. Buffer D was used for all experiments. Binding experiments were conducted using 10 µM DHFR and 100 µM MTX at 25°C. The experimental setup consisted of 19 successive 2 µL injections of either MTX or buffers into DHFR every 180 s to a final molar ratio of 1:2. The first injection was 0.2 µL for all experiments. The data were corrected for the heat of dilution of the titrant. Data analysis was carried out with Origin 5.0 software.