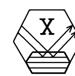


FEBS PLC2016 Ligand-binding course

Academic and University Center, Nove Hrady, July 3 July 10, 2016

Lectures – Monday, July 4

L1

INTRODUCTION TO LIGAND-BINDING THEORY I

Jannette Carey

Department of chemistry, Princeton University, Frick Laboratory, 360 Princeton, NJ 08544, USA

The lectures are intended to equip students with the following skills that can be applied equally to their own binding data or to published data: use the principles of equilibrium, mass action, and mass balance to derive equations describing binding processes; correctly plot binding data; use graphical analysis to interpret binding data; calculate predicted binding isotherms; simulate and fit binding isotherms; determine and quantify affinity, stoichiometry, and cooperativity of binding processes; quantify, diagnose, and evaluate random and systematic errors in binding data. The lectures will include computational exercises.

L2

PROBLEM-SOLVING AND GLOBAL ANALYSIS COMPUTATIONAL EXERCISES

Wei-Feng Xue

School of Biosciences, The University of Kent, Canterbury, Kent, UK

Two workshop sessions will demonstrate the workflow of ligand-biding data analysis. The first workshop will consolidate the basic theory of ligand-biding models and how models are used in binding-data analysis. The second workshop will introduce the concept of advanced global analysis of ligand-binding data. Opportunity will also be available for students to analyse their own protein-ligand binding data.

Lectures – Tuesday, July 5

L3

INTRODUCTION TO LIGAND-BINDING THEORY II

Jannette Carey

Department of chemistry, Princeton University, Frick Laboratory, 360 Princeton, NJ 08544, USA

L4

SURFACE PLASMON RESONANCE

Wei-Feng Xue

School of Biosciences, The University of Kent, Canterbury, Kent, UK

Surface plasmon resonance (SPR) is a powerful method that is capable of resolving not only the affinity of ligand-binding but also the rates of binding relations. Here, principles of SPR will be discussed. How SPR experiments can be set up in practice to measure the association rate, the dissociation rate and the affinity of binding, and how SPR data can be interpreted will also be discussed with emphasise on kinetic models of ligand-binding and global analysis of SPR data.

L5

STUDYING BIOMOLECULAR INTERACTIONS USING ISOTHERMAL TITRATION CALORIMETRY (ITC)

Bruce Turnbull

School of Chemistry and Astbury Centre for Structural Molecular Biology
University of Leeds, Leeds LS2 9JT, UK

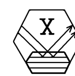
Protein-carbohydrate interactions mediate a wide range of biological processes from signal transduction to viral adhesion and cell invasion. If we are to fully understand such processes, it is important that we can quantify not only the association constant, but also the enthalpic and entropic contributions to the free energy of binding. Isothermal titration calorimetry (ITC) is a sensitive technique for monitoring solution binding thermodynamics that relies on being able to measure the very small changes in heat that arise when two molecules form a complex. ITC can give direct access to G and $T\Delta S$ in a single titration experiment, and under optimal conditions, ITC can also provide information on the binding stoichiometry. If titrations are performed at several different temperatures, it is also possible to determine the change in specific heat capacity (C_p), which is often correlated to changes in buried apolar surface area on binding. In this lecture, i will first introduce how the isothermal titration calorimeter works, and then discuss the relationship between the shape of the titration curve and the observed binding affinity, enthalpy change and receptor concentration. Competition binding experiments will be described as a method for extending the technique to higher and lower affinity systems than can be studied easily using direct titrations. Finally we will consider other influences on the enthalpy of binding, including changes in protonation and heat capacity, and why these factors must be considered carefully when analysing binding thermodynamics. The lecture will be illustrated with examples drawn from ITC studies of the interactions between cholera toxin B-subunit and both its high affinity carbohydrate ligand (ganglioside GM1), and low affinity oligosaccharide fragments.

Useful references:

1. **W. B. Turnbull**, “Divided we fall? Studying low affinity fragments of ligands by ITC”, *G. E. Life Sciences Application Note.* **2011**, http://bit.ly/ITC-Turnbull.

2 **W. B. Turnbull**, B. L. Precious and S. W. Homans, “Dissecting the cholera toxin-ganglioside GM1 interaction by isothermal titration calorimetry”, *J. Am. Chem. Soc.* **2004**, *126*, 1047-1054.

3 W. B. Turnbull and A. H. Daranas, “On the value of *c*: can low affinity systems be studied by isothermal titration calorimetry?”, *J. Am. Chem. Soc*. **2003**, *125*, 14859-14866.



L6

SOME LIKE IT HOT: BIOMOLECULAR ANALYTICS USING MICROSCALE THERMOPHORESIS

David Witte

Nanotemper Technologies GmbH, München Germany

The analysis of bio-molecular interactions and their quantification in the early stages of the drug discovery allows faster development of therapeutics and diagnostic techniques. Here we present Microscale Thermophoresis (MST), a novel immobilization-free and label-free technology for fragment based screening and analysis of the affinity of interactions, such as protein-protein, protein-nucleic acid or protein-small molecule interactions. MST analyzes the directed movement of molecules in optically generated microscopic temperature gradients for instance in complex bioliquids, such as cell lysates and blood serum. The thermophoretic movement is determined by size, charge and entropy of the hydration shell around the molecules. Virtually all interactions and biochemical processes are related to a change in at least one of these parameters upon binding and thus are detectable by MST. MST allows to measure multiple parameters of interactions like binding constants, binding sites, aggregation and binding energetics. Furthermore, MST assays are highly adaptable to fit to the diverse requirements of different biomolecules, e.g. membrane proteins to be stabilized in solution. Also the type of buffer and additives can be chosen freely. This makes MST a highly versatile and efficient technique to study any kind of bio-molecular interactions.

Lectures – Wednesday, July 6

L7

ABSORBANCE SPECTROSCOPY: SEEING IS BELIEVING

Andrea Bellelli

Department of Biochemical Sciences "A. Rossi Fanelli", Sapienza University of Rome, Italy

UV/Vis absorbance spectroscopy is a powerful tool to detect the binding of a ligand to a protein; however it may be abused, leading to wrong determinations. In this the following subjects wil be considered:

(i) the nature of absorbance changes and their (putative) relationship to ligand binding
(ii) the problem of "linearity" of the signal or lack thereof
(iii) the single wavelength analysis according to the law of Lambert and Beer
(iv) a matrix approach to multiple wavelength analysis.

Some real examples are presented, but most of the analysis will be carried out on simulated data.

Selected reference by the author

Bellelli A, Brunori M. (1994) Optical measurements of quaternary structural changes in hemoglobin. Methods Enzymol.; 232: 56-71.

Antonini G, Bellelli A, Brunori M, Falcioni G. (1996) Kinetic and spectroscopic properties of the cyanide complexes of ferrous haemoglobins I and IV from trout blood. Biochem J.;314: 533-540.

L8

FLUORESCENCE SPECTROSCOPY FOR LIGAND BINDING

Andrzej Gorecki

Department of Physical Biochemistry, Faculty of Biochemistry, Biophysics and Biotechnology, Jagellonian University, ul. Gronostajowa 7 30-387 Krakow, Poland

Fluorescence spectroscopy is greatly sensitive to small changes in microenvironment of chromophores. Since the formation of the protein-ligand complex most often disturbs the close vicinity of some chromophores, it is possible to find spectroscopic parameters that identify the apo and holo state of the proteins. For many reasons fluorescence spectroscopy techniques are the most commonly used to investigate protein-ligand interaction. Steady-state and time-resolved fluorescence spectroscopy can be applied to investigate protein-ligand interactions with the use of both intrinsic and exogenous fluorophores. Fluorescence spectrum analysis, fluorescence anisotropy, fluorescence quenching or FRET phenomenon can be used to characterize the thermodynamics, kinetics and structure of the interaction. Physical basics will be presented together with the applications of the techniques and data analysis.

L9

ANALYSIS OF PROTEIN-PROTEIN AND PROTEIN-LIGAND INTERACTIONS BY MASS-SPECTROMETRY METHODS

Rita Grandori

Department of Biotechnolog and Biosciences, Universit of Milano-Bicocca Milan, Italy

Mass spectrometry (MS) has developed into a central tool of biochemistry and structural biology. Such an advancement takes advantage of mild desolvation/ionization techniques that allow preservation of non-covalent interactions while detecting small molecules and bio-p olymers by MS. These techniques conjugate the exceptional analytical power of MS with structural description and, therefore, are particularly well suited to the investigation of complex biochemical systems. This contribution will focus on the basic principles and the main methodological approaches for the analysis of protein-protein and protein-ligand interactions. Examples will be discussed concerning the issues of stoichiometry, specificity, induced folding and relative affinity.

Lectures – Thursday, July 7

L10

ENTROPIC MECHANISMS OF ALLOSTERY

David Dryden

School of Chemistry, Universiry of Edinburg, The King's Builidngs, EdinburghE9H 3JJ United Kingdom

The title of my Ph. D thesis in the mid-1980's was "Functional consequences of protein dynamics". The main part of the thesis was the development of a theoretical model for the involvement of protein dynamics and entropic changes in the phenomenon of allostery. This was followed by a search for experimental proof of this "Allostery without conformational change". I will describe the simple coarse-grained model, the experiments which I attempted to prove the model and some recent work recapitulating and extending the model.

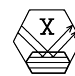
L11

SEDIMENATION ANALYSIS IN ANALYTICAL ULTRACENTRIFUGE

Ondřej Vaněk

Department of Biochemistry, Faculty of Science, Charles University, Hlavova 2030, 12840 Prague, Czech Republic
ondrej.vanek@natur.cuni.cz

Sedimentation analysis of macromolecules carried out in analytical ultracentrifuge is a powerful method for the study of proteins, nucleic acids and other polymers and their various complexes. Monitoring sedimentation of macromolecules in the centrifugal field allows their hydrodynamic and thermodynamic characterization in solution, i.e. in native conditions, without interactions with any matrix or surface. This allows direct measurement of molecular weight and sedimentation coefficient of macromolecules, monitoring of sample purity and homogeneity, prediction of size and shape of sedimenting species and, last but not least, study of equilibrium reactions, including determination of their stoichiometry and equilibrium constants. In the present lecture, we will first focus on the history physical principles of the technique and also on the properties and potential of a modern instrumentation. Two types of experiments performed using analytical ultracentrifuge (i.e. sedimentation velocity and sedimentation equilibrium) will be discussed, together with a brief introduction into sedimentation theory. In the end, sedimentation data analysis will be described (possible hands-on tutorial in computer classroom) and examples of utilization of analytical ultracentrifugation in biomolecule research will be provided. Combination of new instrumentation and computational software for data analysis has led to major advances in characterization of proteins and their complexes. After temporary silence in the past decades, analytical ultracentrifugation at presence experiences renaissance in proteomic and structural biology research while still being heavily used e.g. for characterization of aggregation of monoclonal antibodies in biopharmaceutical industry.



L12

COOPERATIVITY, ALLOSTERY, SYMMETRY, AND LINKAGE

Andrea Bellelli

Dipartimento di Scienze Biochimiche "A. Rossi Fanelli", Sapienza Universita' di Roma

Cooperative ligand binding is a fundamental function of a large number of proteins, whose physiological relevance spans from transport, catalysis and regulation of the cell cycle. The reversible oxygen combination to hemoglobin is a prototype of cooperativity and has been convincingly demonstrated to be a consequence of allostery, the ability of the protein to adopt either of (at least) two structural and energy states. The simplest theoretical framework that correlates cooperativity and allostery is the two-state model originally proposed by Monod, Wyman and Changeux (MWC) in 1965, whose critical assumption is that of perfect structural symmetry.

Angelucci et al. One ring to bind them all (on the concept of extended isologous interfaces)

Antonini et al. 1982 (on the heterotropic effects in Hb)

Baldwin and Chothia (on the structure of Hb and the allosteric structural change)

Bellelli and Brunori BBA (on the mechanism of Hb cooperativity)

Bohr Hasselbalch and Krog (the discovery of homotropica and heterotropic effects)

Monod Wyman and Changeux (the allosteric model; the original definition of the isologous interfaces)

Wyman 1948 (on chemical linkage)

Wyman 1964 (on chemical linkage)

Lectures – Friday, July 8

L13

STRUCTURAL MECHANISMS OF DRUGS STUDIED BY INPHARMA-NMR: METHODOLOGY AND APPLICATIONS

Teresa Carlomagno

BMWZ and Institute of Organic Chemistry, Leibniz University Hannover, Germany,
NMR-based Structural Chemistry Group, Helmholtz Centre for Infection Research, Braunschweig, Germany

Small molecules play a fundamental role in the regulation of the function of proteins, nucleic acids and molecular machines. The development of specific binders that selectively alter the function of only one or a few cellular targets relies on the availability of structural information for the target active site and its mode of interaction with low affinity ligands, identified for example in screening experiments. When this structural information is not available, the rationale design of a selective drug is impossible and the process of drug development has to rely on the screening of large libraries of molecular fragments accompanied by many, lengthy, parallel routes of chemical synthesis.

Our lab developed an NMR-based methodology, INPHARMA [1, 2], which provides access to the relative binding mode of low-affinity ligands to a common target. The method is based on the observation of interligand, spin diffusion mediated, transferred-NOE data, between two ligands A and B, binding competitively and weakly, to a macromolecular receptor T. In accordance with existing SBDD workflows, the experimental information derived from the INPHARMA NOEs is used to select the correct binding mode among many possible binding orientations obtained by molecular docking [3]. The method requires a small amount of non-isotope-labelled target and is widely applicable to different receptor classes.

In this lecture, I will explain the principle of the method, its validation in standard drug design projects, its application to the "induced-fit" case [4] and its expansion to INPHARMA-STRING. In addition, I will demonstrate its usefulness on the basis of several applications [5].

1. V. M. Sanchez-Pedregal *et al.*, *Angew. Chem.* . **44**, 4172 (2005).
2. M. Reese *et al.*, *Angew. Chem.* **46**, 1864 (2007).
3. J. Orts *et al.*, *Angew. Chem.* **47**, 7736 (2008).
4. L. Skjćrven *et al., J. Am. Chem. Soc.* **135**, 5819 (2013).
5. J. Sikorska *et al., Med. Chem. Comm.* **6**, 1501 (2015)

L14

NUCLEIC ACID NMR FOR LIGAND BINDING

Peter Lukavsky

CEITEC Kamenice 753/5 625 00 Brno Czech Republic

Post-transcriptional regulation of gene expression is based on regulatory RNA elements which determine the protein sequence of a gene product through alternative pre-mRNA splicing and control temporal and spatial pattern of protein synthesis in the cytoplasm. Our aim is to unravel molecular principles governing post-transcriptional regulation of gene expression. Using NMR spectroscopy as our main structural tool, we study RNA-protein interaction networks regulating alternative splicing and RNA-protein assemblies crucial for translational control.

I will discuss how TDP-43 mediates mis-splicing of CFTR exon 9 which is associated with severe forms of cystic fibrosis and how the protein Staufen recognizes dsRNA in a sequence-specific manner to regulate mRNA stability. Our mechanistic and structural insights shed light on the complexity of post-transcriptional regulation of gene expression and illustrate how subtle changes on the RNA level can lead to disease.

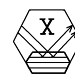
L15

THE MULTIPLE APPLICATIONS OF THE ELECTROPHORETIC MOBILITY SHIFT ASSAY (EMSA) TO STUDY PROTEIN-DNA INTERACTIONS

D. Charlier

Research Group of Microbiology, Vrije Universiteit Brussel

Sequence-specific, structure-specific and non-specific protein-DNA interactions are an integral part of numerous vital cellular processes involving DNA transactions such as replication, repair, modification, recombination, compaction and transfer of DNA, gene transcription etc. Protein-DNA interactions are responsible for the execution, steering and regulation of this wide variety of processes. The electrophoretic mobility shift assay (EMSA) is a widely applied method to study protein-DNA interactions. Its popularity is due to the simplicity of use and high sensitivity. The basic principle of EMSA relies on the resolution of protein-bound (single or multiple complexes) and unbound DNA molecules by migration in native conditions through a matrix (agarose or acrylamide gel electrophoresis) under the influence of an electric potential. Many factors, including mass, charge and shape of the molecules, as well as the migration conditions (composition and concentration of gel matrix, ionic strength, pH, etc.) will affect on their resolution and relative migration distances. As any other technique, EMSA has its strengths and shortcomings. The method is most frequently used for qualitative purposes, but when it is applied correctly, the technique may also provide quantitative data. Furthermore, EMSA may also be a first step in a more in depth analysis of the binding site and sequence specificity such as in-gel footprinting and pre-modification binding interference. In this lecture advantages and shortcomings of the technique will be reviewed and it will be discussed how EMSA can be used to determine apparent equilibrium dissociation constants, binding specificity, the number of binding sites, the stoichiometry of the interaction, binding cooperativity, the effect of allosteric co-factors on the protein-DNA interaction, and intrinsic and protein induced DNA deformations.



Lectures – Saturday, July 9

L16

OVERVIEW OF THE PRACTICAL METHODS

Jannette Carey

Department of chemistry, Princeton University, Frick Laboratory, 360 Princeton, NJ 08544, USA

L17

LIGAND BINDING IN MODERN DRUG DISCOVERY

Preston Hensley

SystaMedic, Inc, New London, USA

The goal of any branch of molecular biology, or human physiology more broadly, is an understanding the relationship between structure and function. Both terms can have a broad and a narrow definition. In drug discovery, since most of our targets are proteins, the goal is relating protein structure to protein function. The simplest function is ligand binding, and ligands can be proteins or small molecules. Fortunately, we have at our disposal a number of high resolution biophysical tools which provide exquisite sensitivity in this analysis. Applications include basic characterization of proteins used in high throughput compound screening and a more high resolution analysis that might be used in new chemotype selection. It is important that these technologies be used in combination, first to establish the basic steps of the interaction and then to use that model to do a complete kinetic and thermodynamic analysis. When such an analysis is accomplished, one can have confidence in the results and in their application to subsequent steps in the drug discovery process.

Exercises

E1

DETERMINATION OF THE EQUILIBRIUM BINDING ISOTHERM OF FLUORIDE AND FERRIC MYOGLOBIN

Andrea Bellelli

Dipartimento di Scienze Biochimiche “A. Rossi Fanelli”, Sapienza Universita’ di Roma

Horse heart myoglobin will be dissolved in Tris Buffer and diluted to 5 to 50 uM. Absorbance spectra will be collected in the visible range (650-500 nm) and Soret (460-400 nm). The sample will then be titrated with stepwise additions of sodium fluoride from a 0.5 M solution and the absorbance spectra will be collected after each addition. The fractional ligand saturation will be calculated from the absorbance spectra and plotted as a function of fluoride and the experimental data will be fitted to a hyperbola using a non linear least squares minimization routine.