



# FEBS Advanced Course

## Ligand-binding Theory and Practice

Academic and University Center, Nove Hrad, June 29 - July 6, 2014

### Lectures – Monday, June 30

L1

#### LIGAND-BINDING THEORY AND PRACTICE

**Jannette Carey**

*Department of chemistry, Princeton University, Frick Laboratory, 360  
Princeton, NJ 08544, USA  
jcarey@princeton.edu*

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 pre-

dicted 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.

### Lectures – Tuesday, July 1

L2

#### ENTROPIC MECHANISMS OF ALLOSTERY

**David Dryden**

*School of Chemistry, University of Edinburgh, The King's Buildings, Edinburgh EH9 3JJ  
United Kingdom  
david.dryden@ed.ac.uk*

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.

L3

#### HEMOGLOBIN ALLOSTERY

**Andrea Bellelli**

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

*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 corre-

lates 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*.

Crucial questions on the structure-function (i.e. on the allostery-cooperativity) relationship in hemoglobin have been thoroughly addressed in the past with reference to the MWC model and provide quite a consistent picture,



whose key elements will be reviewed in the lecture. These are: (i) the *quaternary constraint*, and its structural bases; (ii) the *demonstration of two quaternary structures*, to be correlated with two energy states, irrespective of ligation; (iii) the relationship between structure, oxygen affinity and *dissociation of the tetrameric molecule into dimers*; (iv) the structural and functional characterization of *ligation intermediates*; (v) the *direct demonstration of the equilibrium between allosteric conformations*, by means of rapid kinetic techniques.

Open questions on the details of the structure-function relationship in hemoglobin remain, and suggest that the MWC model needs extensive refinements: e.g. it provides an unsatisfactory description of allosteric effects, and is not consistent with the experimentally determined time course of oxygen dissociation; moreover metastable allosteric conformations have been observed that seem to

violate the symmetry principle. Some of these findings may be reconciled with the model advocating some form of *energy degeneracy* within the low affinity allosteric conformation, but others would require major changes on which no agreement has been found yet.

#### Selected references by the author

Bellelli A, Brunori M, Miele AE, Panetta G, Vallone B. (2006) The allosteric properties of hemoglobin: insights from natural and site directed mutants. *Curr. Protein Pept. Sci.* 7: 17-45 (reprinted and updated in 2013).

Bellelli A. (2010) Hemoglobin and Cooperativity: Experiments and Theories. *Curr. Protein Pept. Sci.* 11: 2-36.

Bellelli A. and Brunori M. (2011) Hemoglobin allostery: variations on the theme. *Biochim. Biophys. Acta (Bioenergetics)*, 1807, 1262-1272.

L4

## THE USE OF MOLECULAR SIMULATION TO PREDICT AND INTERPRET LIGAND BINDING DATA: METHODOLOGICAL ISSUES, STRUCTURAL AND THERMODYNAMIC ASPECTS

Wilfred F. van Gunsteren

Laboratory of Physical Chemistry, Swiss Federal Institute of Technology, ETH, 8093 Zurich, Switzerland

Computer simulation of the dynamics of bio-molecular systems by the molecular dynamics technique yields the possibility of describing structure-energy-function relationships of molecular processes in terms of interactions at the atomic level. Yet, the time and spatial scale of simulations is limited due to finite computing power. Recent advances in simulation methodology, e.g. enveloping distribution sampling (EDS), to compute relative free energies of different states of a system or of different systems can be exploited to rapidly compute many free energy differences based on optimising the sampling of the relevant parts of configurational or conformational space. Methodology to calculate free energy differences is reviewed and its strengths, weaknesses and limitations are demonstrated by application to a variety of bio-molecular systems.

[www.igc.ethz.ch](http://www.igc.ethz.ch) and [www.gromos.net](http://www.gromos.net)

Christ et al., *J. Comput. Chem.* 31 (2010) 1569-1582.

Riniker et al., *J. Phys. Chem.* B115 (2011) 13570-13577.

Riniker et al., *J. Computer-Aided Mol. Des.* 26 (2012) 1293-1309.

Riniker et al., *J. Chem. Phys.* 135 (2011) 024105.

Hansen et al., *J. Comput. Chem.* 33 (2012) 640-651.

Dolenc et al., *J. Computer-Aided Mol. Des.* 25 (2011) 709-716.

Riniker et al., *Helv. Chim. Acta* 93 (2010) 2318-2325.

Steiner et al., *J. Comput. Chem.* 32 (2011) 1801-1812.



## Lectures – Wednesday, July 2

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  $G$ ,  $H^\circ$  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

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

Rita Grandori

*Department of Biotechnology and Biosciences, University 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 biopolymers 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.



L7

## SOME LIKE IT HOT: BIOMOLECULAR ANALYTICS USING MICROSCALE THERMOPHORESIS

David Witte

*Nanotemper Technologies GmbH, München*

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 – Thursday, July 3

L8

## FLUORESCENCE-BASED BIOMOLECULAR INTERACTION MEASUREMENTS

Catherine A. Royer

*Rensselaer Polytechnic Institute, Troy NY USA*

Fluorescence, due to its sensitivity and versatility, is widely used as a means to observe and quantify interactions between biomolecules. There are several possible fluorescence observables and multiple fluorophores, hence providing innumerable strategies for designing the experiment which is most appropriate for the biomolecular sys-

tem at hand. I will briefly define the observables and their properties: intensity, color, anisotropy, lifetime, fluctuations and FRET. The advantages and disadvantages of a number of popular fluorophores will be examined. Then via a few examples, I will discuss how to design and carry out a successful fluorescence-based binding assay.

L9

## ELECTRONIC SPECTROSCOPY

Andzrej Gorecki

*Department of Physical Biochemistry, Faculty of Biochemistry, Biophysics and Biotechnology, Jagiellonian University, ul. Gronostajowa 7 30-387 Kraków, Poland*

Electronic spectroscopy is greatly sensitive to small changes in microenvironment of chromophores. Since the formation of the protein-ligand complex most often disturbs 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 Circular Dichroism and fluorescence spectroscopy techniques are the most commonly used to investigate protein-ligand interaction. Circular Dichroism can be used to determine the secondary and tertiary structure of proteins, which can alter

during the 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 together with the applications of the techniques and data analysis will be presented.



L10

## LIGAND BINDING STUDIED BY INFRARED SPECTROSCOPY

Andreas Barth

Department of Biochemistry and Biophysics, Stockholm University, Sweden

The lecture discusses the use of infrared spectroscopy [1,2] for the study of structural distortions of ligand and protein upon binding. Two proteins serve as examples: the sarcoplasmic  $\text{Ca}^{2+}$ -ATPase and pyruvate kinase. Because the changes in infrared absorption are small, a special approach is needed to detect them with high accuracy [3,4]. This approach implies that the reaction of interest is triggered directly in the infrared cuvette and two techniques for doing this will be presented. By monitoring the conformational change of the  $\text{Ca}^{2+}$ -ATPase, it was possible to identify the functional groups of the substrate ATP which are important for binding [5-7]. Isotopic labelling can be used to detect the signals of the bound substrate depend on the strength of interaction between ligand and protein and on the structure of the bound ligand. For both proteins investigated, structural distortions of the ligand were detected, which however were not directed towards the transition state structure [8,9]. The conformational change of pyruvate kinase upon phosphoenolpyruvate binding depended on the metal ion [10]. In particular,  $\text{Mn}^{2+}$  caused larger conformational changes than e.g.  $\text{Mg}^{2+}$ . This indicates a larger proportion of enzymes in the closed conformation with the former ion. The allosteric effector fructose biphosphate affected this proportion for some combinations of mono- and divalent ions, but not for others [11].

1. A. Barth (2007), *Biochim. Biophys. Acta - Bioenergetics* 1767, 1073-1101, DOI: [10.1016/j.bbabi.2007.06.004](https://doi.org/10.1016/j.bbabi.2007.06.004) Infrared spectroscopy of proteins.
2. A. Barth, C. Zscherp (2002), *Quart. Rev. Biophys.* 35, 369-430, DOI: [10.1017/S0033583502003815](https://doi.org/10.1017/S0033583502003815) What vibrations tell us about proteins.
3. C. Zscherp, A. Barth (2001), *Biochemistry* 40, 1875-1883, DOI: [10.1016/10.1021/bi002567y](https://doi.org/10.1016/10.1021/bi002567y) Reaction-induced infrared difference spectroscopy for the study of protein reaction mechanisms.
4. A. Barth, C. Zscherp (2000), *FEBS Lett.* 477, 151-156, DOI: [10.1016/S0014-5793\(00\)01782-8](https://doi.org/10.1016/S0014-5793(00)01782-8) Substrate binding and enzyme function investigated by infrared spectroscopy.
5. M. Liu, A. Barth (2003), *J. Biol. Chem.* 278, 10112-10118, DOI: [10.1074/jbc.M212403200](https://doi.org/10.1074/jbc.M212403200) Mapping interactions between the  $\text{Ca}^{2+}$ -ATPase and its substrate ATP with infrared spectroscopy.
6. M. Liu, A. Barth (2003), *Biophys. J.* 85, 3262-3270, DOI: [10.1016/S0006-3495\(03\)74744-4](https://doi.org/10.1016/S0006-3495(03)74744-4) TNP-AMP binding to the sarcoplasmic reticulum  $\text{Ca}^{2+}$ -ATPase studied by infrared spectroscopy.
7. M. Liu, A. Barth (2004), *J. Biol. Chem.* 279, 49902-49909, DOI: [10.1074/jbc.M408062200](https://doi.org/10.1074/jbc.M408062200) Phosphorylation of the sarcoplasmic reticulum  $\text{Ca}^{2+}$ -ATPase from ATP and ATP analogs studied by infrared spectroscopy.
8. M. Liu, M. Krasteva, A. Barth (2005), *Biophys. J.* 89, 4352-4363, DOI: [10.1529/biophysj.105.061689](https://doi.org/10.1529/biophysj.105.061689) Interactions of phosphate groups of ATP and aspartyl phosphate with the sarcoplasmic reticulum  $\text{Ca}^{2+}$ -ATPase. An FTIR study.
9. S. Kumar, A. Barth (2010), *Biophys. J.*, 98, 1931-1940, DOI: [10.1016/j.bpj.2009.12.4335](https://doi.org/10.1016/j.bpj.2009.12.4335) Phosphoenolpyruvate and  $\text{Mg}^{2+}$  binding to pyruvate kinase monitored by infrared spectroscopy.
10. S. Kumar, A. Barth (2011), *J. Phys. Chem. B*, 115, 6784-6789, DOI: [10.1021/jp201862a](https://doi.org/10.1021/jp201862a) Effects of ions on ligand binding to pyruvate kinase: Mapping the binding site with infrared spectroscopy.
11. S. Kumar, A. Barth (2011), *J. Phys. Chem. B*, 115, 11501-11505, DOI: [10.1021/jp206272x](https://doi.org/10.1021/jp206272x) The allosteric effect of fructose biphosphate on muscle pyruvate kinase studied by infrared spectroscopy.

## Lectures – Friday, July 4

L11

## THE MULTIPLE APPLICATIONS OF EMSA TO STUDY PROTEIN-DNA INTERACTIONS

Danny Charlier

Research group of Microbiology, Vrije Universiteit, Brussel

Protein-DNA interactions are of utmost importance for the execution, steering and regulation of a wide variety of cellular processes of fundamental importance such as DNA

replication, repair and recombination of DNA, transcription etc. The electrophoretic mobility shift assay (EMSA) is a rather simple and sensitive method to detect and study



these interactions. The basic principle 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) 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 ap-

plied correctly, it may also provide quantitative data. 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.

**L12****UNRAVELLING SEQUENCE-SPECIFIC PROTEIN-DNA INTERACTIONS****D. Charlier***Research group of Microbiology, Vrije Universiteit Brussel*

Proteins may bind DNA in a sequence-specific, structure-specific (or a combination of these) or random manner. Random binding is for instance involved in the compaction of pro- and eukaryotic genomes by nucleoid associated proteins. Examples of exquisite structure-specific recognition can be found in various processes such as the binding of the sliding clamp at the primer:template junction in DNA replication or branch migration and resolution of the Holliday (4-way) junction in DNA recombination by the RuvAB and RuvC proteins.

However, here the focus will be on sequence-specific binding. The recognition of a short specific target sequence (frequently not more than 15-20 base pairs) among the millions of bp of a cell's genome is a crucial aspect of the onset and the control of numerous cellular processes involving DNA transactions, such as origin recognition in bacterial DNA replication and promoter selection and transcription regulation.

Techniques that are used to study sequence-specific protein-DNA interactions can be divided in two major classes: protection studies and premodification binding interference studies. In protection studies the protein is first allowed to bind to the DNA, then the DNA sequence stretch protected by the protein against enzymatic or chem-

ical cleavage is revealed by gel electrophoresis of the reaction products in denaturing conditions. DNaseI, hydroxyl radical and 'in gel' copper-phenanthroline footprinting will be discussed. In the premodification binding interference approaches target DNA is first chemically modified, then it is evaluated how these modifications affect on the protein-DNA interaction. This involves protein binding to the heterogeneous mixture of sparingly modified DNA molecules and their separation on basis of the affinity for the protein in an EMSA, recovery of free and bound forms from the gel, cleavage of the phosphodiester bond at the modified positions and resolution of the reaction products by gel electrophoresis in denaturing conditions. Premodification binding interference studies allow the identification of phosphates, bases or base-specific groups that contribute significantly to complex formation within a zone of interaction with the protein. Modifications may be of different kinds and be backbone-specific (e.g. phosphate ethylation) or base-specific (e.g. groove-specific purine methylation, thymine oxidation, base removal, (missing contact probing) etc.). Various techniques will be discussed and analyzed, including advantages and shortcomings, and experimental data will be interpreted.

**L13****LIGAND-BINDING THEORY AND PRACTICE****Jannette Carey**

*Department of chemistry, Princeton University, Frick Laboratory, 360  
Princeton, NJ 08544, USA  
jcarey@princeton.edu*

NMR in studies of ligand binding. The focus will be on applications of NMR to studies of molecular interactions rather than on NMR methods.