Proteins in Action

biophysical techniques for protein research

Faculty of Science, University of South Bohemia in České Budějovice

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Monday, June 26, Session I

L1

PROTEIN PREPARATION FOR BIOPHYSICAL STUDIES

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The production of high quality protein samples is crucial in obtaining meaningful biophysical data. In contrast to nucleic acids, proteins do not share an appreciable number of common traits, which makes the production and purification of active and well defined samples of the protein of interest quite challenging and unique: One-size-fits-all strategies are seductive but often lead to failure. Knowledge about the overall purity of the protein sample, as estimated by for example SDS-PAGE gel analysis, is by far not sufficient. The best possible starting point for any given biophysical study is to the know answers to basic questions such as, domain organization (*in-silico* analysis), purity (SDS, IEF, SEC), solubility and aggregation state, enzymatic activity, pH optimum and pI, temperature optimum, to name a few.

The aim of the lecture will be to sensitize the students for the topic by addressing a few important aspects such as the Hofmeister series and the solubility phase diagram, themes that are often neglected or underestimated.

Primers

I. A. McPherson, *Crystallization of biological macromolecules*, Cold Spring Harbor Laboratory Press.

II. S. Iwata, *Methods and results in crystallization of membrane proteins*, International University Line Biotechnology series.

III. J. Drenth, *Principles of Protein X-ray Crystallography* (Third Edition, Chapter 16), Springer Science+Business Media LLC.

L2

DETECTION OF FUNCTIONAL PROTEOLYTIC ENZYMES USING ZYMOGRAPHY

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Proteolytic enzymes, also called peptidases or proteases, hydrolyze proteins into smaller fragments (peptides) and often further into single amino acids. Peptidases are present in all living organisms from viruses to vertebrates where maintain many crucial biological processes (eg digestion, wound healing and remodeling or antigen presentation). Peptidases are often parts of a complex protein mixture and the association of peptidolytic activity to a specific type of peptidases can be problematic. To distinguish among the various peptidases, their proenzymes and/or mature enzymes as well as among the different enzymatic isoforms in any biological sample, zymography can be used. Zymography serves as a fast, simple and sensitive electrophoretic technique, which allows us to study the hydrolytic activity of any protein sample on the basis of substrate degradation.

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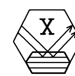

L3

WHAT TO DO IF THE PROTEIN "DOES NOT COOPERATE"

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Preparation of pure proteins in a sufficient amount and desired quality is a crucial step in structure-function studies. The main problems encountered on the way from the gene expression to protein structure determination are related to protein solubility, homogeneity and crystallizability.

A variety of methods have been developed for protein production, purification, etc., however, all steps and conditions have to be optimized with respect to the properties of each individual protein. Thus, the protein itself can be considered as one of the parameters and can also be optimized. Possible approaches and strategies for modifying proteins, e.g. design of single amino acid mutations or fusion proteins, using the methods of bioinformatics and molecular biology will be discussed.

L4

BIOMOLECULAR INTERACTIONS STUDIED BY REAL-TIME LABEL-FREE SPR TECHNOLOGY

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Very little happens in any biological system unless two or more molecules come together to form a stable complex [1]. When molecules interact through specific molecular contacts, all of the principles of thermodynamics, dynamics, and biomolecular structure and recognition come into play. As increasing numbers of new proteins and DNA sequences are entered into databases such as SWISSPROT or GenBank, rapid methods to accurately characterize these biointeractions are needed. One useful model to consider involves a target molecule (T) with a specific binding site (such as a particular region in a protein tertiary structure or a specific sequence of DNA) and a probe molecule (P) that can bind to that site. The simplest binding model corresponds to P + T C, where C is the resulting complex. Probe molecules can vary from small metabolites or drugs to large transcription complexes, and their interactions with the target range from the highly specific (P binds a single site) to the nonspecific (P binds most sites in the target

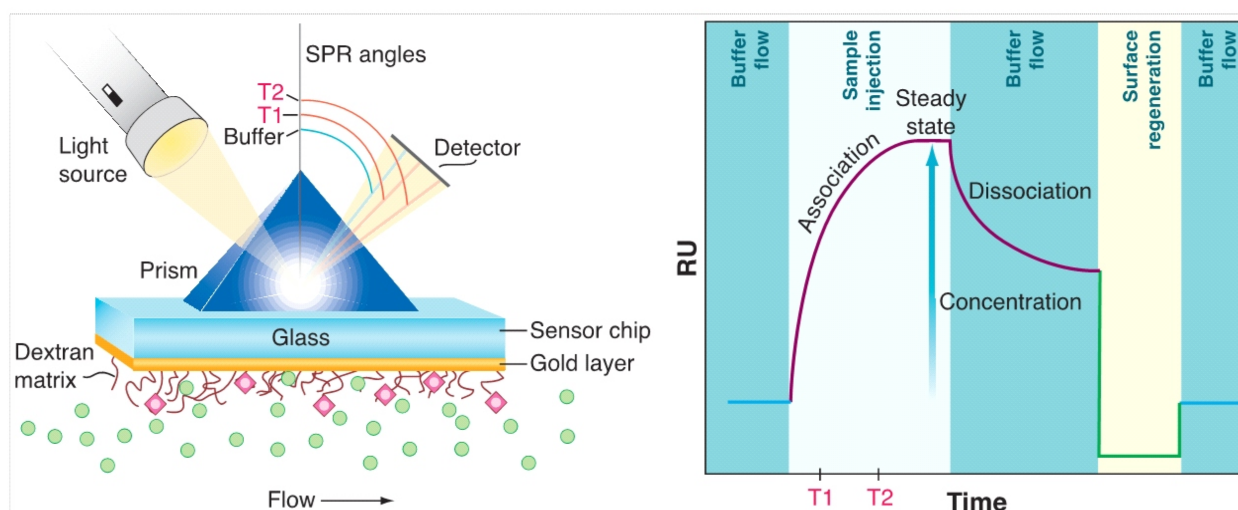

Figure 1: Illustrated SPR. At left, an SPR optical unit and a sensor chip detect the P molecules (green spheres) in the flow solution, which passes by the T (pink diamonds) linked to the dextran matrix. The blue SPR angle defines the position of the reduced-intensity beam. Time points T1 and T2, shown in the schematic sensorgram (right) correspond to the two red SPR angles, which shift as P binds to T over time. As the concentration of bound P increases (arrow), the RU response approaches saturation. The complex dissociates upon reintroduction of the buffer. As shown, the response to the injection solution will fall below the baseline if its refractive index is lower than that of the buffer [Figure adapted from reference 1, Wilson et al.].

class, such as related DNA sequences). In interaction processes that are complicated, there can be multiple binding sites, cooperative interactions, and so forth [1-3]. In order to determine the equilibrium and/or kinetic constants for binding, all techniques must factor the concentrations of P and T into the concentrations of free P and T, on the left side of the equation, as well as the concentrations in C, on the right side of the equation. This evaluation can be achieved by various methods, including equilibrium dialysis, spectral measurements, gel shift, calorimetry, DNase I footprinting, and related techniques [1-4]. Many of these methods require labeling of P or T with a fluorescent or radioactive tag.

A recent development in instruments that investigate biomolecular interactions in label-free mode, is surface plasmon resonance (SPR) detection with a biospecific sensor chip [1-4]. In BIACORE technology, the sensor chip is created by applying a thin layer of gold (~50 nm) to a glass surface (Figure 1, left). In the most common type of sensor chip, carboxymethyl-dextran is linked to the gold to give the interaction layer (~100 nm thick). One of the interacting molecules, either T or P, must be linked to this layer to create the biospecific recognition surface. Because SPR responds to changes in refractive index (Figure 1, right) and, thus, to changes in mass, it is advantageous to attach the molecule with the lowest molecular weight to the surface.

1. W. David Wilson, Science. 295, 2103-5 (2002), and references therein.
2. C. R. Cantor, P. R. Schimmel, Biophysical Chemistry (Freeman, New York, 1980), vol. III.
3. L. G. Fagerstam et al., J. Chromatogr. 597, 397 (1992), and references therein.
4. BIAtechnology Handbook, (BIACORE AB, Uppsala, Sweden, 1998)

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Monday, June 26, Session II

L5

THERMOFLUOR ASSAY: HOW TO MAKE YOUR PROTEIN HAPPY

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Thermofluor assay, also known as differential scanning fluorimetry or thermal shift assay, is a fluorescence-based biophysical method used to assess protein thermostability. Temperature gradient is applied on protein sample in the presence of a hydrophobic fluorescent dye, which binds to the hydrophobic core of the protein exposed during the unfolding process, and fluorescence signal is recorded. Melting temperature, determined from the protein melting curve, is a measure of protein stability in the given environment.

Thermofluor was originally developed for high-throughput screening for ligands [1], but the application of this versatile technique is not limited to drug discovery. It can be used for monitoring of protein-protein interactions [2], assessment of protein-ligand affinity [3], identification of protein function [4], evaluation of properties of protein constructs in engineering [5], or optimization of purification procedure [6].

Thermofluor assay is a key technique of structural biology [7]. Identification of buffer conditions or additives stabilizing the protein prior to crystallization greatly improves the hit rate in initial crystallization screening trials. Thermofluor-based buffer optimization can also give rise to alternative crystal forms with improved diffraction quality. Application of thermofluor will be demonstrated on example cases.

1. Pantoliano *et al.* (2001) High-density miniaturized thermal shift assays as a general strategy for drug discovery, *J. Biomol. Screen.* **6**, 429-40.
2. Kopec & Schneider (2011) Comparison of fluorescence and light scattering based methods to assess formation and stability of protein-protein complexes, *J. Struct. Biol.* **175**, 216-23.
3. Matulis *et al.* (2005) Thermodynamic stability of carbonic anhydrase: measurements of binding affinity and stoichiometry using ThermoFluor, *Biochemistry.* **44**, 5258-66.
4. Carver *et al*. (2005) Decrypting the biochemical function of an essential gene from Streptococcus pneumoniae using ThermoFluor technology, *J. Biol. Chem.* **280**, 11704-12.
5. Lavinder *et al*. (2009) High-throughput thermal scanning: a general, rapid dye-binding thermal shift screen for protein engineering, *J. Am. Chem. Soc.* **131**, 3794-5.
6. Mezzasalma *et al.* (2007) Enhancing recombinant protein quality and yield by protein stability profiling, *J. Biomol. Screen.* **12**, 418-28.
7. Ericsson *et al.* (2006) Thermofluor-based high-throughput stability optimization of proteins for structural studies, *Anal. Biochem.* **357**, 289-98.