Approaches to the Conformational Analysis of Biopharmaceuticals

Roger L. Lundblad

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Series Preface

The universe of biopharmaceutical development revolves around the use of protein science to develop and characterize diagnostic and therapeutic products. Protein Science is a series of books addressed to the application of protein science to biotechnology. Applications of protein science include site-specific chemical modification; spectroscopy and spectrophotometry; electrophoresis; hydrodynamic analytical techniques such as viscosity, light scattering, and analytical ultracentrifugation; chromatographic fractionation including size-exclusion chromatography; and expression and purification systems. This series is directed at the practitioner of commercial biotechnology so there will be volumes on drug product classes including plasma protein products, monoclonal antibodies, cytokines and chemokines, and receptor proteins. To the extent that the series is focused on commercial biotechnology, there will be less discussion of "cutting-edge" and more discussion of direct application of proven new technologies to the study of protein therapeutics. The various volumes will be published in a timely manner to assure immediate value to the biotechnology community.

Preface

This book has been designed to gather as much of the technology for the conformational analysis of biopharmaceutical polymers as possible into a single volume. It is not intended to cover the use of a specific technology in detail. Rather, it is intended to provide sufficient information and references to establish the basis for the selection of a specific experimental approach that would be the most cost-effective in the study of biopolymer conformation. Thus, coverage of some technologies is brief since they have little application to biotechnology products; this is not meant to suggest, however, that such technologies are not of great value in basic science studies. There is also an emphasis to describe studies where multiple technologies were used to address conformational change.

The next several years will see increased interest in the conformational analysis of biopharmaceutical polymers resulting from the development of biosimilar or "follow-on" biological products. The activity of many biopharmaceutical polymers is dependent on conformation. Thus, a comparison of a "generic" (off-patent) biological medicinal product with the originator product includes conformational analysis. A clear understanding of what any differences between products mean or do not mean is critical; thus, the need for independent methods of analysis. It is hoped that this book will emphasize the need for science over hype, the need for rational analysis rather than "smoke and mirrors." Also, renaming a technique does not increase its value in any way.

Finally, I would be remiss if I did not thank Professor Bryce Plapp of the University of Iowa for his continued support of the thermodynamically challenged. Professor Charles Craik of the University of California at San Francisco has also provided some useful advice as has Professor Don Gabriel of the University of North Carolina at Chapel Hill. I am also most indebted to Jill Jurgensen and Barbara Norwitz of Taylor & Francis for their support.

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Roger L. Lundblad received his undergraduate education at Pacific Lutheran University, Tacoma, Washington, and his PhD in biochemistry from the University of Washington, Seattle. After postdoctoral work in the laboratories of Stanford Moore and William Stein at the Rockefeller University, New York, he joined the faculty of the University of North Carolina at Chapel Hill in 1968. Dr. Lundblad joined the Hyland division of Baxter Healthcare in 1990. Currently, he is an independent consultant and biotechnology writer based in Chapel Hill, North Carolina. He is an adjunct professor of pathology at the University of North Carolina at Chapel Hill and an editor in chief of the *Internet Journal of Genomics and Proteomics*.

Contents

1 Introduction to Biopharmaceutical Conformational Analysis: Issues and Methods

Most biopharmaceuticals are proteins or protein conjugates and are considered to be biopolymers. Proteins have a unique conformation in solution, which is a product of diverse covalent and noncovalent interactions. It is generally accepted that the primary structure of proteins dictates the secondary and tertiary structure of that protein and the final conformation is stabilized by the aforementioned covalent and noncovalent interactions. These interactions can be intramolecular or intermolecular; intramolecular interactions dominate at low protein concentration while intermolecular interactions are more significant at higher protein concentration where such forces are involved in processes such as aggregation. That is not to say that intermolecular interactions are not important at low protein concentrations; however, such interactions are usually driven by specific multivalent interactions.[1]

The study of protein conformation has been of great interest for the study of the relationship between structure and function[3,4] for some time and for the study of protein folding.[5,6] The emergence of biosimilars in commercial biotechnology[7–14] has increased interest in the use of protein conformation study in comparability studies.[15–18] Comparability is also of importance when there are process changes, formulation changes, and change in source material.[19–25]

Protein conformation is the combination of secondary structure (helix, pleated sheet)[26–30] and tertiary structure.[31–37] It is generally accepted that primary structure drives secondary structure, which in turn drives the formation of tertiary structure.[38–40]

The characterization of a protein therapeutic is a critical part of the development and drug approval process. Classic methods such as sequence analysis, compositional analysis, solution behavior with particular emphasis on the formation of aggregates and, more recently, analysis by mass spectrometry are used in the evaluation of protein structure as a biopharmaceutical. The question then is what quality attributes are critical for product performance and what physical/chemical techniques would effectively measure these attributes. It is generally accepted that immunogenicity is a most significant problem. Issues with glycosylation, which influence product half-life and may influence immunogenicity, are also of importance. The problem of immunogenicity is discussed below and elsewhere in detail[41–46] as are techniques for the

evaluation of glycosylation.[47–53] Glycosylation presents a little amount of challenge: while glycosylation is important for circulatory half–half (specifically the covering of galactose/galactosamine by sialic acid), there is precious little evidence to suggest a true functional role for glycosylation.

Most solution protein chemistry characterization assays for biologics have focused on chemical structure and biological activity. There is somewhat less interest in the use of conformational analysis. There are several reasons for this. First, to a certain extent, conformational analyses for purposes of identity or comparability only are useful if there is no change: if there is change, it is usually, but not always, difficult to quantitate as compared, for example, to a chemical modification in the peptide chain. However, there are a variety of techniques that can be used to study protein conformation.[54]

Analytical techniques such as amino acid analysis and mass spectrometry provide information regarding the chemical structure of the product. Techniques such as electrophoresis, chromatography, and size exclusion chromatography provide information about purity and can, in selected situations, provide insight into conformation and chemical structure. Hydrophobic interaction chromatography[55–59] can also be useful in the study of conformational changes in proteins.[60–67]

The past 40 years have provided an increase in the sophistication of the technologies available to measure conformational change in proteins; there has not been an increase in the parameters measured. Kauzmann[68] proposed a classification system for the levels of conformation similar to the general classification of primary, secondary, tertiary, and quaternary structure, which separated conformation issues into shape properties and short-range properties. Shape properties (long-range) were parameters dependent on the overall shape (globular, rod, etc.), which might be relatively insensitive to changes in the immediate vicinity of amino acids and peptide bonds. Short-range properties include parameters defined by the immediate environment around individual amino acid residues. Granted that this is an imperfect separation, it does prove useful. Schellman and Schellman[3] reviewed the problem of conformation change in proteins in 1964 and as observed by Cantor and Timascheff,[69] there had been no change in the some 20 years between the two reviews. There has been a marked increase in the sophistication of the instrumentation, and Schellman and Schellman extended Kauzmann's earlier suggestions. Shape properties included hydrodynamic parameters such as frictional coefficient and viscosity changes and solution properties such as fluorescence depolarization and flow birefringence. Also included in shape is electron microscopy, dipole moments, and diffusion through controlled pore membranes (thin film dialysis).[70–72] Short-range properties are, to some extent, "micro" properties as compared to the "macro" properties of shape. Schellman and Schellman include optical properties such as absorbance (IR, UV) and circular dichroism and chemical properties such as side chain reactivity (trace labeling, chemical footprinting), individual pK_a's, hydrogen isotope exchange, biological activity, and immunogenicity as short-range properties. Also included in short-range properties are nuclear magnetic resonance (NMR) and binding of small molecules such as dyes. This division is admittedly imperfect; for example, immunogenicity could be more accurately defined as a shape property but reactivity is dependent on epitopic change.

Most of the techniques used for the conformational analysis of protein were developed either for the study of protein denaturation or, more recently, for the study of protein folding. The focus of this chapter is directed toward the application of solution protein chemistry to the study of conformational change associated with the processing of biotechnology products. These changes can be considered more closely related to denaturation than to protein folding. Denaturation[73] can be considered to be phenomena associated with the change in the spatial arrangement of the polypeptide chains in a protein (tertiary structure) from the native, ordered structure to a more disordered structure in an irreversible process. Denaturation is usually, but not always, associated with loss in solubility. Denaturation is not usually associated with the cleavage of the peptide chain. There are, however, situations that seem to be a slight exception to this; the conversion of fibrinogen to fibrin and the cleavage of peptide chains. Protein denaturation is frequently, but not always, associated with the loss of biological activity as it has long been accepted that configuration is important for biological activity.[74,75] Protein denaturation is not necessarily irrereversible[76–81] but there can be a divergence in the quality of structure recovery dependent on measurement.[82–84] The key to renaturation is, in part, dependent on the quality of protein; for example, while some zymogens (e.g., pepsinogen) can be reversibly inactivated under conditions where active enzymes (e.g., pepsin) are irreversibly inactivated.[85–87] On the other hand, trypsin can be reversibly denatured[88,89] and may be more stable than trypsinogen to denaturation.[90–92] Techniques such as light scattering[93–98] and analytical ultracentrifugation[99–104] provide information about the shape and solution behavior of the material (tertiary structure and quaternary structure). These two techniques together with size exclusion chromatography are critical for the evaluation of aggregation in pharmaceutical preparations. There is also reason to consider measurement of the second virial coefficient. The second virial coefficient is a factor used to correct for the nonideal behavior of a particle. Virial coefficients were originally developed as a series of coefficients of inverse powers of V in a polynomial series to approximate the quantity of pV/RT in an equation of state of an ideal gas or similar collection of particles.[105,106] From a practical perspective, the second virial coefficient is related to the excluded volume of a particle[107,108] and is important in accounting for protein–protein interactions and molecular crowding.[109–112] The excluded volume of any particle depends on shape and can be defined as the volume surrounding and including a given object, which is excluded to another object.[107] The second virial coefficient is mentioned most often in the study of the osmotic pressure of proteins but has general use for the study of protein–protein interaction.[113–127] The reader is recommended to articles on protein shape,[128–132] as this attribute is frequently overlooked in favor of the more sophisticated approaches discussed below.

Techniques such as circular dichroism,[133–140] optical rotatory dispersion,[69,141–147] Fourier transform infrared spectroscopy (near infrared [NIR]),[148–157] NMR,[158–170] intrinsic fluorescence,[171–182] binding of fluorescent probes,[183–192] hydrogen–deuterium exchange,[193–207] differential scanning calorimetry,[208–224] Raman spectroscopy,[225–241] protein footprinting,[242–247] limited proteolysis,[248–265] and trace labeling[266–269] can provide information about secondary and tertiary structure. NIR spectroscopy is also useful for noninvasive determination of moisture.[269–276]

One of the major problems with the use of most of these techniques is the requirement for substantial amounts of protein. This can be an issue with therapeutic proteins, which are biologically active at the microgram level and the use of a destructive analytical technique is difficult to justify. However, the use of mass spectrometry for analysis enhances the sensitivity and therefore the value of hydrogen isotope exchange and trace labeling. An analysis of the literature indicates that optical rotatory dispersion is of limited value today as compared to other analytical technologies.

The key issue is—what is the question that you wish to answer? Each of the various techniques has the potential to show changes in conformation secondary to changes in the solvent environment. However, what is the relationship of these changes to biological activity, in vivo clearance, or immunogenicity? In the case of a biopharmaceutical, if you lose activity, you are losing product. Creation of new epitopes (increase or change in immunogenicity; neoantigenicity) either results in an unfortunate immunological response or increased product clearance.[42,44,277] Changes in the immunological properties of biotherapeutic proteins can be identified by established immunoassays.[45,278–281] Changes in glycosylation such as the loss of sialic acid (exposure of galactose/galactosamine) can also increase the rate of product clearance. The demonstration of conformation change in a protein does not necessarily predict a loss of activity or neoantigenicity but can provide insight into the chemistry responsible for such changes. The material below described the relationship between the chemical modification of a protein and changes in secondary or tertiary structure.

First, while the following discussion emphasizes changes in protein conformation (secondary and tertiary structure), primary structure and quaternary structure should be briefly considered. Changes in primary structure can be divided into two categories: first, the modification of individual amino acid residues, which is covered in great detail in Chapter 18 and, second, the cleavage of peptide bonds mostly by proteolytic enzymes or chemical means (i.e., cleavage at asparagine[282–286]). Changes in primary structure will be discussed as such changes in primary structure influence secondary and tertiary structure; changes in quaternary structure are in turn driven by changes in secondary and tertiary structure. As practical note, this author is not aware of any biotherapeutic proteins that were displaying a quaternary structure as an issue; however, general issues of protein–protein interaction, which are important for quaternary structure, are important in the action of most protein biotherapeutics.

Protein conformation can be influenced by both physical and chemical agents. One of the more lively discussions 40 years ago concerned the effect of site-specific modification on protein conformation with respect to the elucidation of the relationship between chemical structure and biological function. Without belaboring the detail, it was generally accepted that it was possible to accomplish the site-specific chemical modification of a protein without gross conformational change, but it was always useful to evaluate such potential changes.[287–289] This has been an active area of interest with the PEGylation of proteins.[290–293]

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