

Tools for Protein Structure Characterization

Classic Biochemical Procedures Are Experiencing a Renaissance

K. John Morrow Jr., Ph.D.

A formidable array of techniques allows researchers to describe the properties of protein molecules. While nucleic acids are much more restricted in their structural variations, the universe of possible combinations for the 20 basic amino acids permits virtually unlimited variation in proteins. Powerful hardware including mass spec, x-ray crystallographic, and NMR machines have morphed dramatically into user-friendly companions while providing a wealth of descriptive data.

In some cases, this surfeit of information can confuse and retard analyses, wasting time and resources. For this reason, there is renewed interest in applying classic biochemical methods in novel ways to provide structural information concerning protein molecules of interest. Collectively, such innovative applications may provide decisive evidence regarding the utility of a protein for therapeutic use.

Many of these protein-characterization tools have been around since the early days of biochemistry. But, the refinement of automated and robotic instrumentation has greatly simplified their application, as has the introduction of many premade and disposable options.

Macromolecular Characterization

"Light scattering is the only practical method that takes the ambiguities out of measurement of molecular weight and size," said Geoffrey Wyatt, president of

Wyatt Technology (www.wyatt.com). Wyatt developed the first multiangle laser-based commercial instruments 40 years ago, he claimed, and since that time has pioneered many light-scattering hardware and software advances.

The firm's technology is based on the principle that molecules in a solution will scatter a beam of light at all angles. The greater the mass and/or concentration of the molecules, the greater the amount of light scattered. This so-called static light scattering measures the intensity of the scattered light as a function of angle. This property is known as Rayleigh scattering and can yield information on the mass, size, shape, and structure of the molecules under scrutiny without making a priori assumptions about the composition of the molecules.

Because the size of a molecule affects the angular variation of the scattered light intensity, an instrument fitted with detectors at a plurality of angles can determine the angular dependence of the light-scattering event. Small molecules will produce little or no angular variation in the scattered light, whereas larger molecules (for instance, a random coil of about 50 kD) will cause a signal that allows determination of the mean square radius of the molecule directly.

When the vertically polarized laser-light beam is passed through a sample, the intensities of the scattered light at all angles have a response directly proportional to the mass of the molecules (times their concentration). These analog signals are then digitized and processed by a computer in order to yield unambiguous molar masses,



Galapagos reports that it has developed a number of innovative protocols to generate proteins in sufficient purity and quantity for investigating protein interactions.

sizes, and their distributions.

Another common light-scattering approach is known as dynamic light scattering (or quasi-elastic light scattering); it takes advantage of the fluctuations of light intensity occurring on a microsecond or millisecond scale, normally measured at a single angular location (usually 90°). These fluctuations are a function of the Brownian motion of the molecules in solution, which in turn, is related to the hydrodynamic radius of the molecule. Smaller molecules have greater molecular motility, e.g., they move faster in solution than larger molecules.

The dynamic light-scattering instrument actually counts the photons of light being scattered using a special multimode optical fiber. By analyzing the correlation function, one can directly measure the diffusion constant of the molecule, from which the molecule's equivalent hydrodynamic radius can be determined. Even molecules that are not spherical are assumed to be using dynamic light scattering.

According to Wyatt, the light-scattering technology complements conventional 1-D and 2-D gel analysis of proteins to yield additional insights into biomolecular behavior. When the molecular weight of molecules is determined from acrylamide gels, it is necessary to denature the molecules by boiling them under reducing conditions, which profoundly alters their structure. The fact that light-scattering instruments produce data on native molecules provides an important comparison in many investigations.

Cleaving Antibody Genes

Antibody genes undergo shuffling and rearrangement in order to generate the diversity that is the hallmark of the immune response. This scrambling of antibody gene sequences is the result of lymphocytic events in which a family of enzymes clips out and sutures this information in order to produce new combinations of antibody-coding sequences.

Karla Rodgers, Ph.D., professor in the

department of biochemistry and molecular biology at the University of Oklahoma Health Sciences Center, and her colleagues are grappling with two enzymes, RAG1 and RAG2, that catalyze the first cleavage steps in the recombination process of the variable antibody gene family. According to Dr. Rodgers, the RAG proteins contain 500–1,000 amino acids, with a core and a noncore region. The core region is composed of the sequences for DNA recognition and catalyzes the cleavage of the DNA.

To delineate the role of zinc in the functioning of the cleavage enzymes, Dr. Rodgers' team employed atomic absorption spectroscopy, a long-standing approach that reveals the levels of a wide range of different metals in a biological sample. The technology, defined in the nineteenth century, was thoroughly explored during the 1950s by a team of Australian chemists. It makes use of a flame to atomize the sample, turning a liquid sample into an atomic gas. Typically, the solvent is evaporated, and the remaining dry sample is vaporized into a gaseous phase and then broken into free atoms.

A beam of light is then passed through this flame and is absorbed by a detector. When high voltage is applied across the anode and cathode, the metal atoms in the cathode are excited into producing light with a certain emission spectrum. The electrons of the atoms in the flame can be driven into higher orbitals for an instant by absorbing a set quantum of energy, defining a specific electron transition in a particular element.

Dr. Rodgers has shown that the Rag-1 and Rag-2 enzymes, both of which are required for DNA cleavage, come together to form a complex with the immunoglobulin gene sequences to catalyze the appropriate slicing of these segments. The role of the zinc atoms was defined through the use of an atomic absorption spectroscopy. The migration patterns of the proteins on non-denaturing gels, the gel-shift assay, identified the DNA cleavage activity of the enzymes. Through the application of mass

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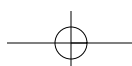
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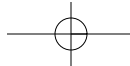
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spec and limiting proteolysis, the Rodgers lab was able to identify the domains of the enzymes and assign functions to them.

In addition, the group used various mutations to pin down the amino acids that are essential to the binding of the zinc to the enzyme. "We concluded that the catalytic region of core RAG1 enzyme binds multiple Zn²⁺ atoms, making it a unique member among this enzyme family," Dr. Rodgers said.

Combining a variety of methodologies has enabled Dr. Rodgers to build an elegant description of the Rag-1 and Rag-2 enzyme family and establish their functions in the process of immunoglobulin-gene recombination. Filling in these gaps allows a rigorous approach for designing new antibody therapeutics.

Shotgun Mutagenesis

Another unique approach to protein characterization does not rely on high- or low-tech instrumentation but rather defines protein structure and function through the effect of mutational changes on the target molecule. According to Cheryl Paes, Ph.D., research scientist at **Integral Molecular** (www.integralmolecular.com), shotgun mutagenesis utilizes traditional mutation analysis, but in a high-throughput manner, to rapidly evaluate the

effects of amino acid substitutions across the entire length of a protein.

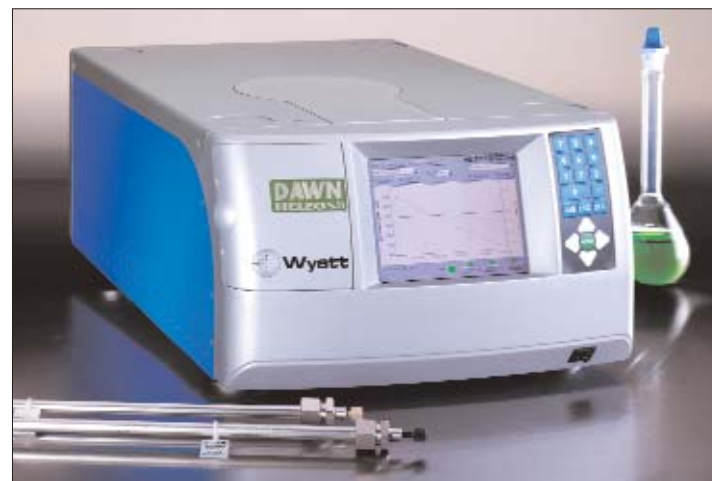
Dr. Paes employs a patented, high-throughput expression technology permitting simultaneous evaluation of thousands of mutations on protein function. A customized plasmid library is created for each target gene, with each clone bearing a unique amino acid substitution. The clones are arrayed individually in microwell plates and assayed for a defined function of interest. Because each clone is sequenced at the time of creation of the library, the critical residues affecting protein function can be mapped onto the protein structure.

This approach is an alternative way to derive information usually gleaned through the use of x-ray crystallography or NMR. Shotgun mutagenesis, claimed Dr. Paes, saves vast amounts of time and resources and offers options for structural analysis of difficult proteins such as GPCRs that could not usually be otherwise achieved.

The company is currently mapping a wide range of interactions with difficult membrane proteins. One project concerns the epitope mapping of mAbs directed against the chemokine receptor CCR5.

A shotgun mutagenesis library of the protein was prepared, composed of point mutations along the entire length of the 1

Wyatt Technology's DAWN® HELEOS™ II is an 18-angle light-scattering detector for the measurement of absolute molecular weight, size, and conformation of macromolecules in solution.



kb CCR5 protein. The effect of each mutation on binding of the mAbs was assessed by immunofluorescence. Comparison of the side chains that could and could not support antibody binding permitted atomic level structural requirements for each antibody to be identified.

Shotgun mutagenesis can also be used to investigate drug interactions with target molecules. In one series of experiments, Dr. Paes' team mapped the binding site of TAK-779, a small-molecule inhibitor to the CCR5 receptor, the principle coreceptor for HIV. The critical amino acids were identified using an HIV infection assay.

The binding site for the TAK-779 drug was identified as point mutations that supported HIV use of the coreceptor but eliminated inhibition by the drug. By using the contact residues as constraints in drug docking, a 3-D binding model of the most plausible drug-target interaction could be constructed.

Adequate Protein Supply

Galapagos (www.glp.com) uses many procedures to delve into protein structure and function, noted Patrick Mollat, Ph.D., protein sciences team leader. In order to pursue these goals, the company has developed a number of innovative protocols to generate proteins in sufficient purity and quantity for investigating protein interactions.

Galapagos believes that studying these interactions will point the way to molecules with therapeutic potential. Dr. Mollat presented some of his research activities at the recent "PepTalk" conference in San Diego. "Each new protein is a challenge," Dr. Mollat stated. "You have to know what is known, and how and what polypeptide you want to express."

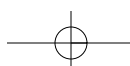
Bone mineralization and resorption, a daunting symphony of interacting pathways, has been one of the company's major interests. Androgen receptors, proteins that are critical in aging males as androgen production declines, are among the many molecular players in this complex network. The aim of the program is to generate tissue-selective androgen receptor modulators that improve bone quality and muscle strength by mimicking the androgens' beneficial anabolic effects on bone and muscle while avoiding the negative risks of prostate cancer exacerbation.

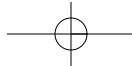
In one series of experiments, Dr. Mollat's group improved the expression of the ligand-binding domain of the androgen receptor in order to produce enough protein for crystallization with the goal of doing x-ray crystallography.

After some unsuccessful preliminary efforts, the team settled on a thioredoxin-6 histidine-ligand binding-domain construct that was expressed in bacteria. Using IMAC columns, they were able to obtain material with a degree of purity sufficient for crystal formation, and with this material, they determined the structures of the two requested ligand-binding domain-androgen receptor-ligand complexes at a 1.9 Å resolution.

A Listing of Technologies and Products for Protein Characterization

Company	Technology	Products	Comments
Advion www.advion.com	LC/MS with simultaneous fraction collector	Characterize targeted PTMs missed by LC/MS	Glycosylation identification
Alliance Protein Lab www.ap-lab.com	Various techniques to measure protein folding, protein stability, protein confirmation, the oligomeric state of the native protein, and protein aggregation	Characterization of oligomeric state and aggregation by two different light-scattering techniques: dynamic light scattering and online classical light scattering used with size-exclusion chromatography	CMO
Applied Biosystems www.appliedbiosystems.com	Mass spec systems	Linear ion trap LC/MS/MS, MALDI-TOF, MALDI TOF/TOF, ESI-TOF, triple quadrupole TOF, and ESI MS/MS	New software for MS performance, methods development, data acquisition, and processing
Arvys Proteins www.arvysproteins.com	Analysis of raw, intermediate, and final products	Electrophoresis (SDS-PAGE, native PAGE, IEF-PAGE, and urea-PAGE) and Western dot blot	Contract studies
Beckman Coulter www.beckman.com	UV/Vis spectrophotometry	ProteomeLab DU 800	Determines protein concentration using commercial assays and enzyme kinetics/mechanism studies
Bio-Rad Laboratories www.bio-rad.com	Classical and innovative protein- and peptide-characterization tools	Instruments and reagents for life science research	Large selection from one of the founders of macromolecular characterization
Blue Stream Laboratories www.bluestreamlabs.com	Analytical capabilities	Protein/peptide characterization including primary and secondary structural analysis and carbohydrate analysis	Contract services
Cirrus Pharmaceuticals www.cirruspharma.com	Protein and peptide characterization	Circular dichroism, electrophoresis, SEC, UV, and DSC	CMO
Covance www.covance.com	Mass spectrometric, chromatographic, and electrophoretic tools	Range of GLP- and GMP-compliant physicochemical and biological techniques	Characterization of protein structure and stability
ExpASY www.expasy.ch	Software for protein characterization from mass spec and other data	Aldente, FindMod, GlycoMod, Mascot, PepMAPPER, PFMUTS, ProFound, ProteinProspector	Available free
Genomic Solutions www.genomic-solutions.com	Automated solutions for genomics and proteomic research	Investigator Proteomic System	Integrates via both hardware and software with mass spectrometers
Malvern www.malvern.de	Protein analysis	Light-scattering spectroscopy, fluorescence, circular dichroism, DSC, NMR, analytical ultracentrifugation, and small-angle x-ray spectroscopy	Contract services
NextGen Sciences www.nextgensciences.com	2-D gel electrophoresis	2DEoptimizer automates gel casting to user-defined gradients: PC control for planning and monitoring 2-D processes, runs high-speed IEF, and casts gradient gels of any dimension	Technologies to automate and optimize 2-D methodology
Paragon Bioservices www.paragonbioservices.com	2-D gel electrophoresis	Includes protein characterization by immunoblots	Contract services
Wyatt Technology www.wyatt.com	Laser-light scattering	New fully automated dynamic light-scattering DynaPro Plate Reader comes with temp. control	Applications highlighted in many peer-reviewed journals





In another project, Dr. Mollat isolated members of the Dickkopf (Dkk) protein family, which bind to one subunit of the Wnt receptor complex. These proteins are poorly understood but play an important role in embryonic regulation, malignancy, and other disease processes. The goal was the production of multiple Dkk proteins for antibody characterization, and Dr. Mollat moved to a mammalian expression system using **Invitrogen's** (www.invitrogen.com) FreeStyle 293-F cells combined with transient transfection. The proteins secreted into the medium were harvested and purified on a nickel IMAC column. By this strategy, 12 different proteins were purified in six months and all were biologically active.

Dr. Mollat has learned a lot from his protein-purification work. "Always adjust the production and purification procedure to the application at hand based on the protein characteristics and the existing information, optimize the expression level from the very beginning of the study, and try to find the simplest way to do the work."

Understanding Proteins

In analyzing protein structure and function, clearly there is no one-size-fits-all approach. Information collected through the use of shotgun mutagenesis provides insights into the 3-D configuration of proteins, bypassing the time-consuming and expensive process of crystallizing and x-ray mapping a target. Yet the approach may not provide the fine resolution gained through the use of traditional x-ray crystallography.

"While I believe that the approach of mutagenesis is interesting and can give information about the residues that are important for the activity or the stability of your protein," opined Dr. Mollat, "I feel that crystallographic analysis gives more precise information, particularly regarding the mechanism of action of compounds."

For many of the properties that affect the behavior of proteins, the traditional, low-tech approaches including 1-D polyacrylamide gel electrophoresis can be rapid, direct, and highly informative. Combined with 2-D gel separation and immunoblotting, these inexpensive and technically undemanding techniques can tell the investigator a lot about targets and set up a scenario for bringing in the big guns for the most promising leads.

According to Muctarr Sesay, Ph.D., senior director for process development at **Goodwin Biotechnology** (www.goodwinbio.com), "We find ourselves challenged in both directions as we build a protein-development process. We need to deliver quality that meets regulatory requirements but at the same time, conform to rigid timelines."

It typically costs between \$1 and \$4 million for a CMO to create a master cell bank from a stable research cell bank provided by the client. This entails development of a scalable and compliant process for upstream cell culture and downstream purification including manufacturing and product characterization of antibodies or other therapeutic proteins.

For GMP proteins, the risk for adhering

to compliance grows as development and manufacturing proceed from the upstream to the downstream process, so protein characterization (safety, identity, potency, strength, and purity) is integral.

"We use a wide range of characterization tools, which range from relatively simple analytical assays such as HPLC-SEC, ELISA, SDS-PAGE, and IEF to very elaborate mass spec technology," he continued. "Whereas, early on in preclinical and Phase I a generalized picture of the molecule may

suffice, later stages of manufacturing may require extremely detailed and precise characterization."

This may include light-scattering and sedimentation-velocity studies, which are of critical importance in defining aggregation, absolute molecular weight, and the quaternary structure of large antibodies such as IgMs. Bringing all this technology to bear for a Phase II-III product is challenging and time consuming. During preclinical and Phase I drug manufacturing,

Goodwin may spend a year or more building a complete manufacturing process in order to develop a well-characterized and compliant drug product.

With so many technologies to choose from, investigators may find themselves with an overabundance of options. While application of these powerful tools provides for refined characterization of macromolecules, it also means that early decisions and the correct choice of investigative options are all the more critical. **GEN**

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