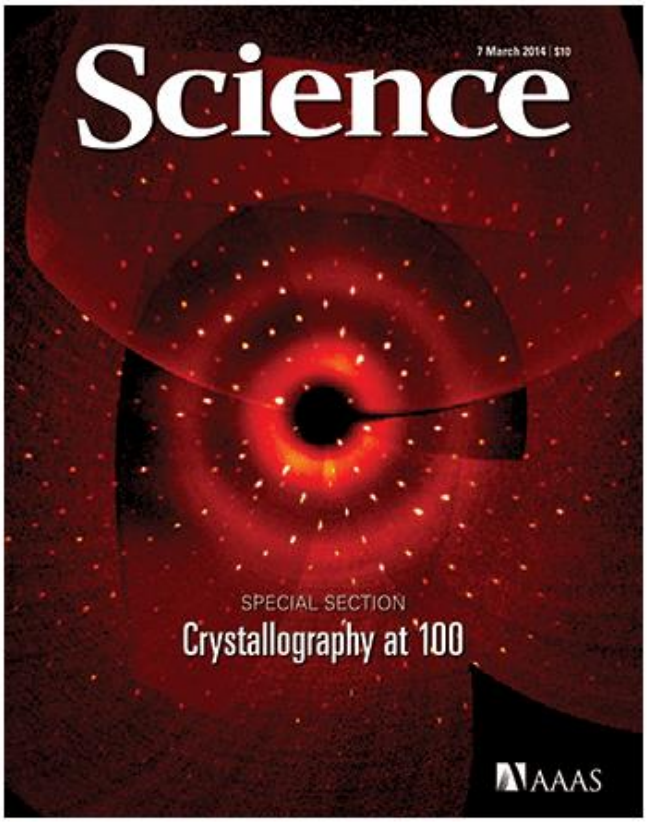
SPECIAL ISSUE | 7 MARCH 2014

Crystallography at 100

Science's Special Issue on Crystallography at 100 celebrates the insights gained from structural studies that have revolutionized our understanding of chemical and biological systems, leading to the award of 29 Nobel Prizes for scientific achievements related to, or involving the use of, crystallography.

About the Cover



COVER Precession image reconstructed from x-ray diffraction data collected from a benzene single crystal. For the past 100 years, x-ray crystallography has been a key tool for determining the structures of ever more complex chemical and biochemical systems. See the special section beginning on page 1091 and at www.sciencemag.org/special/crystallography. Image: Judith A. K. Howard and Michael R. Probert

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INTRODUCTION

Going from Strength to Strength

IN 1912, THE GERMAN PHYSICIST MAX VON LAUE PUBLISHED THE FIRST PAPER demonstrating x-ray diffraction from a crystal. This discovery, for which he was awarded the Nobel Prize in 1914, provided a window into the regular atomic arrangements within crystals. Today, the Cambridge Structural Database contains more than 600,000 structures of organic and organometallic molecules, many obtained through x-ray crystallography; the Protein Data Bank contains about 100,000 structures. The insights gained from these and other structural studies have revolutionized understanding of chemical and biological systems, leading to the award of 29 Nobel Prizes for scientific achievements related to, or involving the use of, crystallography.

In their Review (p. 1098), Howard and Probert highlight advances in studying single crystals of nonbiological molecules and materials. Novel approaches are helping crystallize unstable samples and mount them in the x-ray diffractometer without damaging the fragile crystals. Advanced x-ray sources allow structures to be obtained from smaller crystals and provide access to time-resolved data on chemical reactions within crystals. Crystals can now be studied at low temperatures and high pressures, further extending the range of conditions and samples that can be structurally characterized.

Garman (p. 1102) charts the history of structural biochemistry, from the initial report of x-ray diffraction from pepsin crystals to the recent characterization of the entire ribosome and of G protein–coupled receptors in different conformational states. She discusses the challenges of protein crystallization, which is increasingly automated. The vast majority of protein structures come from synchrotron beamlines, many of which now offer sample-mounting robots, microfocus beams, and the ability to collect supplementary (e.g., spectroscopic) data. Radiation damage may be overcome through the use of x-ray free-electron lasers. In a related Perspective in *Science Signaling*, Smerdon discusses the insights into the regulation of the kinase mTOR gained from protein crystallography.

Building on the success in obtaining static structures, Miller (p. 1108) discusses efforts to capture atomic motions in crystals in real time. Very bright tabletop electron sources have been used to study photoinduced phase transitions and photoinduced organic reactions. Time-resolved x-ray diffraction experiments are mainly performed at synchrotron light sources, although the development of tabletop instruments is under way. X-ray free-electron lasers offer exciting opportunities for time-resolved studies, particularly of biomolecules.

Science's News writers mark crystallography's centenary with a timeline by Sumner (p. 1092) highlighting some of the field's most celebrated discoveries and advances. Service (p. 1094) describes researchers' long quest for the elusive structures of the proteins that act as gatekeepers to cell membranes. Finally, in News Focus (p. 1072), Service reviews the Protein Structure Initiative, a major research program sponsored by the U.S. National Institutes of Health, and looks ahead to how its scheduled shutdown in 2015 could affect structural biology.

– ROBERT COONTZ, JULIA FAHRENKAMP-UPPENBRINK, MARC LAVINE, VALDA VINSON

Crystallography at 100

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REVIEW

Cutting-Edge Techniques Used for the Structural Investigation of Single Crystals

Judith A. K. Howard[1]* and Michael R. Probert[2]

X-ray crystallography has become the leading technique for studying the structure of matter at the atomic and molecular level. Today it underpins all sciences and is widely applied in industry. It is essential in the development of new materials. The technique is very powerful, and the range of materials that can be studied expands as new technologies evolve and are applied in innovative ways to structure solution. It is now possible to record vast amounts of diffraction data in seconds electronically, whereas it took days and months by photographic methods 30 to 40 years ago. Single crystals can be created in various ways; they can be produced from compounds that are liquids or gases at room temperature, and complete molecular structures can be presented within minutes. This short review presents recent developments that are appropriate to the single-crystal x-ray studies of chemical and materials sciences.

One of the most important scientific tools to emerge from the 20th century is x-ray crystallography. Because the chemical and physical properties of a material depend on its structure, the three-dimensional results derived from a crystallographic study are of enormous importance in the overall characterization of any new material. In recent decades, this technique has also revolutionized the understanding of molecular biology. The centenary celebrations in 2013 for the ground-breaking discoveries of W. H and W. L. Bragg (*1*) provide an appropriate point to look at the modern techniques in use today. This short review will concentrate on single-crystal x-ray diffraction methodologies, referencing new instrumentation, sources, and computational tools. We will assume a basic understanding of the single-crystal method and refer the novice reader to some introductory texts on the x-ray experiment for collecting diffraction data (*2*, *3*).

X-ray crystallography experiments have traditionally required single crystals; today, however, there are pioneering studies in the use of multiple crystals (*4*) with methodologies and programs to interpret data recorded from twinned or multicrystal samples (*5*). Samples are no longer required to be crystalline and stable at room temperature, and many single crystals have been grown from liquids by controlled variation of the temperature (*6*) or the careful application of pressure (*7*) (Fig. 1). We shall start by describing some methods for crystallization, linked to the appropriate instrumentation, followed by further instrument and x-ray source developments, and finally explore new computational methods.

Crystallization and Crystal Mounting

Crystals can be grown in the laboratory from solution, by evaporation of the solvent, by cooling, and by balanced-diffusion experiments. The single crystal required for a diffraction study is selected by visual inspection, normally under an optical microscope, from the batch of crystals grown. This crystal is then mounted and supported rigidly during the collection of three-dimensional diffraction data. All crystals are mounted, by various means, onto a goniometer head (Fig. 2), a device with at least three degrees of freedom that allows the crystal to be centered in the x-ray beam. Many methods have been developed for mounting crystals that can be handled in more or less ambient conditions in preparation for the x-ray experiment. Of particular note is the use of perfluorinated oils, which has facilitated fast, reliable mounting of unstable, as well as routine, samples. In this approach, crystals are "fished" from the mother liquor into an oil-filled fiber loop, and thus are suspended in the inert oil. This method is much easier than the previous one of sealing crystals inside thin-walled glass capillary (Lindemann) tubes. The older method, using Lindemann tubes, is still employed if the sample exhibits rapid decomposition through solvent loss. This method allows a local positive pressure of the crystallization solvent to be created, by sealing the tube with the crystal and a drop of the solvent. Crystal mounting techniques are still being developed using ever-more exotic materials, such as graphene, to reduce background during the diffraction experiment.

Crystallization from the melt of a material, by zone refining—a localized heating method—has been employed successfully in producing large single crystals for use as semiconductors—for example, in the electronics industry (*8*). On a laboratory scale, zone refining is useful when dealing with materials that have low melting points and exist as liquids at room temperature (*9*). The laboratory methods used for the growth of crystals from liquids are outlined below. If the crystals are to be grown from liquids by cooling or by pressure, then the container in which the crystal growth takes place is also the mount used for the crystallographic characterization. When crystal growth is to be controlled by temperature, the pure liquid is sealed in a short capillary tube that is mounted in a metal holder and attached to the goniometer head (Fig. 2, inset). This assembly is then placed onto the diffractometer (Fig. 2), the instrument for conducting the diffraction experiment, and the growing procedure can begin. The contents of the capillary are cooled well below its melting point to give a microcrystalline solid or a glass; the temperature is then raised to just below the melting point, and the process of zone refining begins. This requires carefully controlled temperature changes to produce successive melting and crystal growth. The smallest crystals are melted first, and the larger crystals that persist can then act as seeds for the next round of crystal growth. This process is iterated until a suitable single crystal has been achieved. The task requires considerable skill and patience, but the results are most rewarding. The introduction of the optical heating and crystallization device (OHCD) (*10*), a targeted laser that allows highly localized heating while the rest of the sample remains below the melting point, improved success rates in this field. It successfully removed much of the chance present in the more primitive methods, which allowed only those with "gifted hands" to produce diffraction-quality crystals from liquids. Many laboratories now use these methods, producing novel and impressive results. Producing crystals in situ, which may take several hours of optimization, requires careful control of conditions to give a suitable crystal for diffraction.

Crystal growth from liquids can also be initiated by the application of pressure; this is usually achieved inside a diamond anvil cell (DAC) (*11*) (Fig. 3). The approach used is similar to the one described above, whereby the conditions are carefully controlled around the sample's melting point. In this case, however, the liquid is pressurized into a solid state, either microcrystalline or a glass, and then the pressure is controlled around the melting point to initiate crystal growth. If a suitable crystal can be grown successfully, the diffraction experiment must then be carried out with the crystal inside the DAC under the conditions in which it was created, often with pressures exceeding several thousand atmospheres. This method can result in the creation of a different polymorph from that obtained on cooling (*12*). Changes in the protocol for pressurization on the

[1]Chemistry Department Durham University, Durham DH1 3LE, UK. [2]School of Chemistry, Newcastle University, Newcastle NE1 7RU, UK.

*Corresponding author. E-mail: j.a.k.howard@durham.ac.uk

same sample can also induce different polymorphic forms, which cannot be achieved through other methods. There is an unexplored world of crystalline forms waiting to be discovered through careful control of the sample environment using modern techniques. DACs can produce extremely high pressures up to geological scales to simulate pressures in the mantle and in Earth's interior (*13*), but these pressures are rarely approached in chemical studies, because excessive pressure destroys the single crystals grown at more moderate pressures.

Crystal growth under nonambient conditions of pressure or temperature has opened up areas of chemistry in which the only methods for structure characterization previously were the various supporting spectroscopies. In addition, it has led to the discovery of novel polymorphs, some of which had been suggested by structure prediction software (*14*), a field that has recently blossomed with the advent of powerful modern computers. Recent work, attempting to grow cocrystals from combinations of two liquids, has also revealed a further route to undiscovered polymorphs, in cases where only one of the individual components crystallizes rather than the expected cocrystal (*15*).

X-ray Sources and Detectors

Recent advances in both detector technology and x-ray sources have greatly contributed to the ongoing revolution in crystallography. Sources and detectors are intimately combined with the methods and instrumentation and, therefore, are synergic with developments in the field. Enormously brighter x-ray sources; better, faster detection; and advanced crystallographic software all enable and encourage innovative experiments and novel instrument development. The global crystallographic community and commercial companies work closely to ensure that innovative ideas become affordable instruments or devices.

X-ray Sources

Sealed tube x-ray sources were the commercially available laboratory standard until the early 2000s with diffractometers being equipped with either a copper or a molybdenum metal target (anode). These metals produce x-radiation of mean wavelengths 1.5406 and 0.7107 Å, respectively, and are operated at a power of 1.6 to 3 KW. Other metal targets (Sc, Ti, Cr, Mn, Fe, Co, Cu, Mo, Rh, Ag, Gd, W, Au) have become available, but are much less common and are used for more specialized experiments ranging from single-wavelength anomalous diffraction (SAD) phasing to high-resolution, high-pressure studies. Rotating anode generators were developed (*16*) to increase the incident flux at the sample, enabling much smaller crystals to be analyzed by x-ray diffraction. The increase in power was made possible by spreading the heat load on the anode. In these generators, the anode rotates in a vacuum and is internally cooled with water. Rotating anode generators, providing high-brilliance laboratory sources, are now in demand in all areas of crystallography, allowing the study of exotic materials where crystal size is limited (*17*). Microfocus optics, using multilayer components to focus the x-rays, were introduced into laboratory sources more than 10 years ago, but only more recently have they been available in reliable and affordable formats (*18*) and been used routinely by instrument manufacturers for the three most common targets, Cu, Mo, and Ag. The optics are incredibly efficient and, coupled to the latest generation of x-ray tubes, allow high flux densities to be achieved at a fraction of the power of traditional sources (<50 W compared to >6 kW), having a major environmental impact on laboratory energy usage.

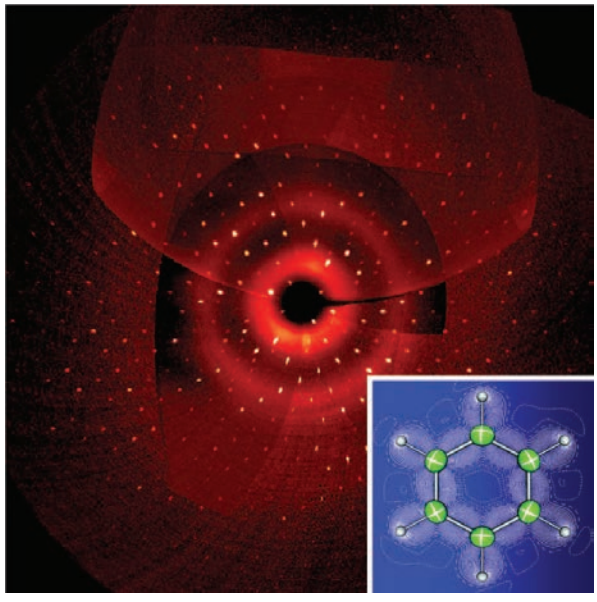

Fig. 1. Precession photograph image and molecular structure of benzene (*28*).

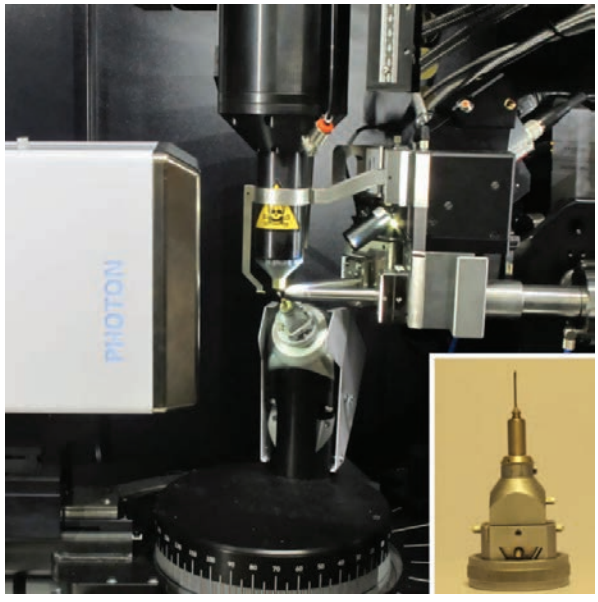

Fig. 2. A modern dual-source, three-circle diffractometer, with large CCD area detector. Goniometer head (**inset**) allows the crystal to be positioned accurately at the center of the instrument for data collection.

In the latest high-intensity laboratory sources, designed for the study of exceedingly small crystal samples, the conventional solid metal anode has been replaced by a liquid metal jet, thus removing the cooling previously required for the anode to be maintained at temperatures well below its melting point. The liquid metal alloy can support a higher electron beam power density than a solid anode, and can therefore generate a much higher x-ray flux. The latest commercially available system uses a liquid gallium-rich (~90%) alloy, coupled with a LaB_6 cathode, producing an x-radiation of wavelength ~1.34 Å. Future developments of this technology are expected to expand the number of available metal alloys for different wavelength applications (*19*).

The advent of synchrotron radiation for dedicated use as a high brilliance x-ray source in the 1960s is now part of our history, but previously there had been some parasitic use of facilities that were designed primarily for the high-energy physics community (*20*). There are now more than 40 large synchrotron facilities across the world, and crystallographers are major users of these facilities. Data collection strategies for chemical and materials crystals are generally less complicated than for protein samples, but the intense beams can cause substantial radiation damage to the crystals, and new protocols have been devised to reduce this.

The most recent advance in the area of x-ray sources is the development of x-ray free-electron laser (XFEL) facilities, which provide short, intense, coherent femtosecond x-ray laser pulses with intensities that are many times higher than in current-generation synchrotron sources. The short wavelength (<1 Å) and pulse lengths provided, from 200 down to 30 fs, will be appropriate to study fast chemical reactions, which are too rapid to be captured by other methods. The Linac Coherent Light Source (LCLS) at SLAC Stanford began operation in 2009, SACLA at Spring 8 became operational in 2012,

and the European XFEL at DESY (Hamburg) will be online in 2015 (*21*). Very recent experiments on photosystem II at the LCLS (*22*) show what can be achieved with XFEL. The emergence of such sources offers very exciting challenges for the future, requiring extremely fast processing and management of vast amounts of data that each experiment will produce on every structure to be studied at these sources in decreasing quanta of experimental beam time. It may, for example, be possible to trigger chemical or biological reactions inside the crystals during their diffraction experiment. Another challenge lies in merging and interpreting data from many crystals that may not all behave in exactly the same way.

Detectors

As x-ray sources became brighter, detector technology necessarily moved in parallel. Huge changes in experimental procedure followed the introduction of charge-coupled devices (CCDs) into laboratory diffractometers in the 1990s. CCDs, the technology in many digital cameras, allow diffraction images from large phosphor sensors to be rapidly recorded, decreasing the time required for a standard diffraction experiment from days to hours. The previous state-of-the-art required each reflection in a diffraction pattern to be measured individually with a single point detector. Area detectors had been in use for some time in neutron diffraction studies (*23*), but only a limited number of protein laboratories were using them for x-ray studies, before the increased demand by the new synchrotron beamlines and subsequent investment in laboratory infrastructure in chemistry and materials science departments. Since then, new developments have brought faster, more sensitive, more reliable detectors with no moving parts into the laboratories and onto facilities' beamlines. The latest developments have introduced solid-state detectors, which allow direct photon counting, continuous readout, and time gating of the detector itself (*24*). Each subsequent development has enabled experiments that were not feasible with the previous generation, up to the present time where entire diffraction data sets can be collected in a matter of seconds.

The demands of XFEL will drive the next generation of fast x-ray detectors and the software to process the vast amounts of data recorded. The detectors and software will bring us closer to the realization of real-time studies of chemical and biological processes, where subsequent experiments can build up direct observations of these events.

Crystallography at Low Temperatures and High Pressures

Routine, low-temperature laboratory data collections were unusual until the 1980s, by which time only 4% of data sets in the Cambridge Structural Database (CSD) (*25*) were shown to have data recorded below room temperature. This has risen to 44% of all structures deposited since 1980; taking deposits since 2000, this number rises to 57%. The introduction of cooling the sample by a stream of cold gas (*26*) expanded the range of samples that could be studied routinely, allowing air-sensitive samples to be mounted in inert oils, which freeze on cooling. It also enabled the collection of crystallographic data from radiation-sensitive crystals (*27*). The reduction in thermal motion and diffuse scattering, the increase in the amount and quality of the data, and the stabilization of sensitive samples are all recognized advantages of low-temperature studies (*28*). The decrease in thermal motion not only improves standard diffraction data quality, but also enables the collection of high-resolution data. These data enable structural studies to move beyond the determination of atomic positions and allow the investigation of a material's full electron density (*29*–*31*), resulting in a greater understanding of its electronic characteristics and physical properties. In addition, it is possible to study subtle structural changes by recording data sets over a wide temperature range. This is of particular interest when structural phase changes can be correlated to the sample's macroscopic properties. Understanding the link between the molecular structure and the property of a material is a fundamentally important aspect of today's multidisciplinary studies.

A number of cooling devices are available for use with single-crystal diffractometers, depending on the temperature range required. The most commonly employed device uses a cold inert gas stream, usually nitrogen, which is directed onto the crystal throughout the experiment to maintain a set temperature. This temperature can be varied at controlled rates across the range of the device—for example, from ~80 to ~500 K for modern open-flow nitrogen systems. These devices are highly successful and the most common in crystallography laboratories across the world. There are also open-flow systems that use helium gas as the crystal coolant (*32*), providing a temperature range from 15 to 300 K. These devices are less widely used owing to cost of operation, but have the great advantage of allowing access to the sub–liquid nitrogen temperature range (below 77 K). Cooling to ultralow temperatures requires a different approach, as open-flow technologies cannot access this region. A desire to understand fundamental solid-state physics phenomena that occur only at these very low temperatures gave rise to the development and use of closed-cycle cryorefrigerators (CCRs), where the sample is cooled by conduction and thermally isolated under vacuum. The use of these systems has not been large outside of central facilities, but these are enormously powerful instruments, providing unique access to this temperature regime (*33*). CCRs impose some experimental constraints due to their size, but allow specialized experiments to record accurate, high-resolution, x-ray data over a very wide temperature range—for example, 2 to 300 K when using a three-stage closed-cycle He gas cryorefrigerator. The instruments are not available commercially; they are expensive and time-consuming to build, but uniquely powerful for determining the three-dimensional structure of materials at very low temperatures and for studying phase changes that relate to important macroscopic properties, such as molecular superconductivity and magnetism (*34*).

DACs, mentioned previously, are most commonly used for high-pressure studies of materials that are crystalline under ambient conditions. They enable the elucidation of structure evolution under pressure variation, analogous to the investigation of structural modifications that accompany changes

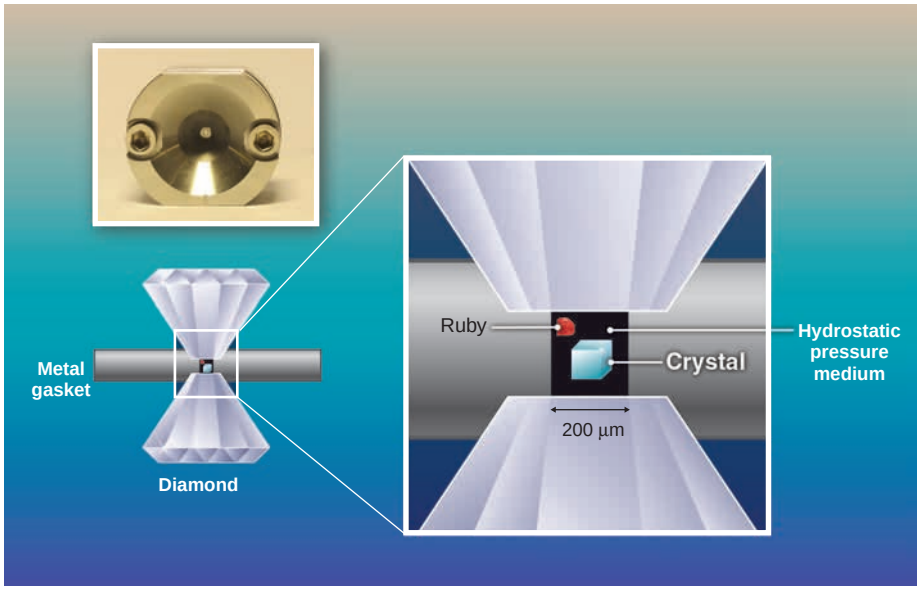

Fig. 3. A diamond anvil cell (DAC) (top left) capable of pressures >100 kbar, with operational schematic (below).

in physical, optical, electrical, or magnetic properties that are followed today over wide temperature ranges (*28*). Unfortunately, the data that can be recorded in a high-pressure experiment using DACs are restricted, because the cell body obstructs the diffracted x-ray beams. Modifications to the original DAC design alleviate some of these problems (*35*), but the diamonds and the bulky superstructure of the cells themselves create an obvious physical limitation. In most high-pressure experiments, a tiny ruby chip is enclosed with the crystal and its hydrostatic medium inside the small gasket of the cell (Fig. 3). This is used to determine the pressure within the cell, as the changes in the fluorescence spectrum of ruby with pressure have been extremely well calibrated. Data reduction requires careful attention because there are strong reflections from the diamonds and scattering from the cell body materials. This has driven the development of programs to apply "masking" to the data set and to correct the data adversely affected by the DAC scattering or diamond reflections (*36*). A portable, moderate quartz pressure cell (QPC) has been designed that uses a moderately thick-walled quartz capillary tube as the pressure chamber to contain an optically visible crystal and to enable single-crystal data collection at pressures of up to 1 kbar created by the application of gas or liquid (*37*). The use of a gas to apply the pressure also enables the investigation under non-ambient atmospheres (*38*–*40*). The QPC system has operational and data-reduction advantages over the DACs, but in a limited pressure range, albeit one that fills the gap between ambient pressure and the lower range of DACs for single-crystal samples.

These experiments have led to the full characterization of materials that exhibit abnormal behavior upon the application of pressure, such as negative compressibility, and also enable the monitoring of pharmaceutical active ingredients under the moderate pressure conditions used during tablet formulation.

Photocrystallography

There is considerable scientific and industrial interest in this area of structural chemistry, which aims to determine the full three-dimensional structure of photoinduced species in order to understand the molecular and macroscopic properties with respect to the ground state and excited states of the material. Mapping often subtle structural changes induced by light, heat, pressure, magnetism, and electric current with respect to time is fundamental to our understanding of reaction mechanisms, but achieving this in the solid state is a considerable challenge. Pioneering work (*41*–*44*) has established the techniques required for these experiments, and we are approaching true "time-resolved" studies with the latest x-ray (XFEL) sources (*45*, *46*). Recent decades have seen an explosion in optical and optoelectronic devices that exploit switchable materials, and it is necessary to understand these molecular and electronic processes in detail to design and create new materials that are stable and robust to thermal/photocycling (*47*). The conversion ratios between states (photoexcited to ground) is commonly rather small in many solid-state reactions, and ways to enhance this for usable materials is one goal of this growing research area.

Photoactivation can be reversible or irreversible, short- or long-lived, and each type of "switch" presents challenges for the crystallographer to achieve high-resolution structures and requires different experimental methodologies. The very fast (femtosecond) chemical reactions require the latest, brightest x-ray sources and very fast lasers, whereas some long-lived reactions can be followed at synchrotron sources when observing in the micro-to-millisecond time frames (*48*). Although much research has been published for decades on photochemical reaction studies by optical spectroscopies, the molecular detail and high atomic resolution of crystallography have been missing. We can now perform the complementary diffraction experiments to enhance our understanding of these fundamental, but highly important, chemical and biochemical processes.

Photoswitchable materials include spin crossover (SCO) compounds (*49*–*52*), photo-(thermo)-chromic materials (*53*–*56*), photocycloaddition (PCA) compounds (*57*, *58*), and photoisomeric compounds (*59*, *60*), and these have found application variously in optical storage materials, light and pressure molecular switches, sensors, molecular wires, logic gates, and imaging (Fig. 4). Photocrystallographic experiments strive to achieve high-resolution diffraction data from the ground state and subsequent excited states in the same single crystal, which requires a conversion of at least 10% and no serious degradation of the crystal in the process of excitation. There are many examples of these successful experiments in the literature, but achieving the reverse process in a single crystal can be challenging or impossible, depending on the chemical reaction.

Crystallographic Software

Structure solution and refinement algorithms have advanced with the increasingly accurate, higher resolution, x-ray data now recorded, largely free of systematic errors. Several structure solution and refinement packages (*61*–*64*) are now available, all being actively developed to interpret more complete structural data and reduce possible errors in the final model. Structural descriptors that go beyond the spherical atom model (*65*), and allow the full electron density elucidation of compounds, have become more mainstream, and further developments in this field now require only moderate-resolution data. The advances in both detector and source technologies outlined above have driven the development of data collection and processing software, with many central facilities using robotic mounting and centering routines (*66*). Data from these are often relayed to automatic processing software that will attempt to produce a near-finished molecular model. Combining these functions moves the more routine structural interrogations into the realm of full automation. This allows the crystallographic experts to concentrate on the more challenging systems, such as multivariable experiments, obscure sample environments, low-resolution data, incommensurate crystals, and quasicrystals. One further area seeing rapid development is in the strategies to record, process, and interpret data from experiments designed to follow reactions in real time (*46*).

A recent revolution in structure solution that is important to mention is the introduction of the charge flipping algorithm (CFA) (*67*). This is a dual-space

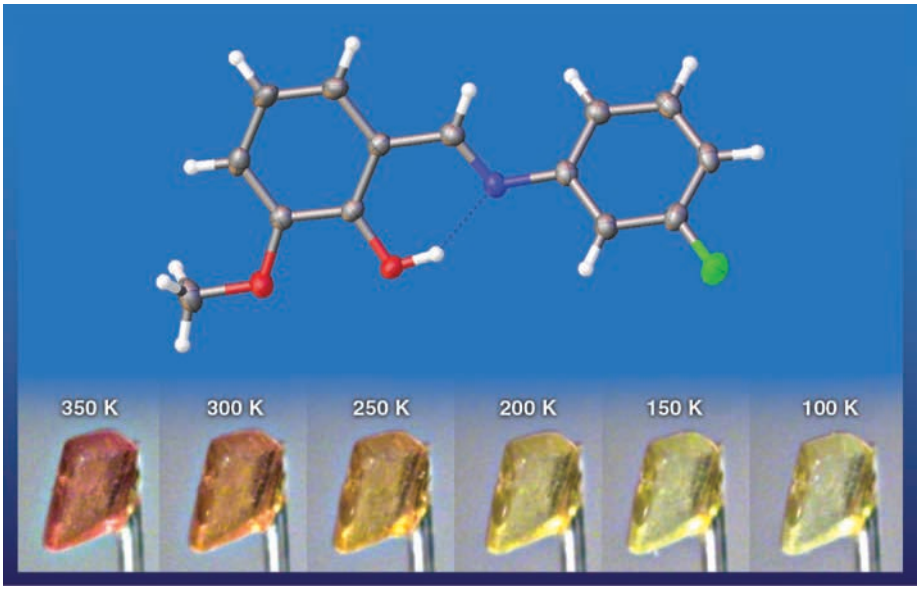

Fig. 4. Molecular compound that undergoes a minor conformational change with temperature. (**Below**) The crystal of the compound, showing obvious thermo-chromic behavior between 100 and 350 K.

phasing algorithm, utilizing the fundamental knowledge that electron density in a crystal structure must be positive. The method has rapidly become a popular alternative for data sets where traditional methods fail (*68*). CFA has a major advantage over traditional solution methods, as the space group of the structure does not need to be determined before use. It is the only structure solution method that is currently extensible to systems where the full symmetry of the system is described by $3 + n$ dimensions.

The Future

Chemical and materials sciences lie at the basis of the next generation of smart materials, fabrics, and devices, and x-ray crystallography is fundamental to their design and successful application. The use of crystallography in online analysis will continue to be an essential industry tool, and instruments will become faster, smaller, more portable, and applicable in the field for important health problems in remote areas and the developing world. Concurrently, the development of new powerful x-ray sources for the laboratory, as well as at global central facilities, will enable new discoveries at higher resolution by using much smaller crystals, and importantly, these experiments will use much less of the crystalline materials in the studies, whether pharmaceutical compounds, precious metals, or the rare chemicals that are needed in modern electronics. Recent discoveries at the molecular level for smart materials with clever magnetic and electrical properties (e.g., single-molecule magnets) require extensive dynamic structural studies to explain the subtle molecular changes under applied external fields so that these changing properties can be exploited in the next generation of devices. Taking crystallography to other planets, most recently Mars, has challenged the imagination of crystallographers, engineers, mathematicians, and many other materials scientists, with staggering results, and we can expect to see more missions that take remote-controlled laboratories to distant places—missions that were unimaginable a few years ago. The collaboration of scientists developing portable x-ray sources, fast, sensitive detectors, intelligent robots, innovative software, and data analysis methods will find many applications and challenges for crystallographers in the decades ahead. Fortunately, crystallography has a long history of sharing ideas, experiences, expertise, methods, and software for the common good (*69*, *70*).

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REVIEW

Developments in X-ray Crystallographic Structure Determination of Biological Macromolecules

Elspeth F. Garman

The three-dimensional structures of large biomolecules important in the function and mechanistic pathways of all living systems and viruses can be determined by x-ray diffraction from crystals of these molecules and their complexes. This area of crystallography is continually expanding and evolving, and the introduction of new methods that use the latest technology is allowing the elucidation of ever larger and more complex biological systems, which are now becoming tractable to structure solution. This review looks back at what has been achieved and forward at how current and future developments may allow technical challenges to be overcome.

Macromolecular crystallography enables the three-dimensional (3D) structures of large biologically interesting molecules to be determined. Structures of proteins and nucleic acids determined by macromolecular crystallography are vital for elucidating protein function

and intermolecular interactions and for improving our understanding of basic biological and biochemical mechanisms and disease pathways. Their immediate practical application is in the design of pharmaceuticals, in which they play a central role in drug discovery.

This branch of crystallography has dramatically advanced over the past 80 years since the 1934 initial observation of diffraction from crystals of a small protein, pepsin, and the first protein structure determination (myoglobin) (Fig. 1A) in 1958. Haemoglobin followed, and then in 1965 the first enzyme structure, lysozyme (Fig. 1B), was solved. The recent characterization of the entire ribosome (Fig. 1C) revealed one of the essential machines of life, comprising a vast complex of molecules consisting of ~280,000 nonhydrogen atoms: more than 2.5 orders of magnitude larger than the 1260 in myoglobin. The field has been awarded 28 Nobel Prizes—starting with father-and-son team William Henry and (William) Lawrence Bragg in 1915—with the latest being the 2012 Chemistry Prize won by Kobilka and Lefkowitz for studies on G protein–coupled receptors (GPCRs), crucial cellular sensors for signaling proteins and hormones. These Nobel Prizes signal the effect that crystallography has had and continues to have in the world of cutting-edge research.

Macromolecular crystallography was born with the pivotal discovery by Bernal and Crowfoot (*1*) that pepsin crystals retained their order if kept hydrated in a capillary tube sealed at each end during x-ray diffraction experiments. Unlike the crystals formed by inorganic or small organic compounds, macromolecular crystals can contain up to 90% solvent surrounding the molecules. The intermolecular interactions supporting the crystalline lattice are weak. The success of diffraction experiments

Department of Biochemistry, University of Oxford, South Parks Road, Oxford OX1 3QU, UK.
E-mail: elspeth.garman@bioch.ox.ac.uk

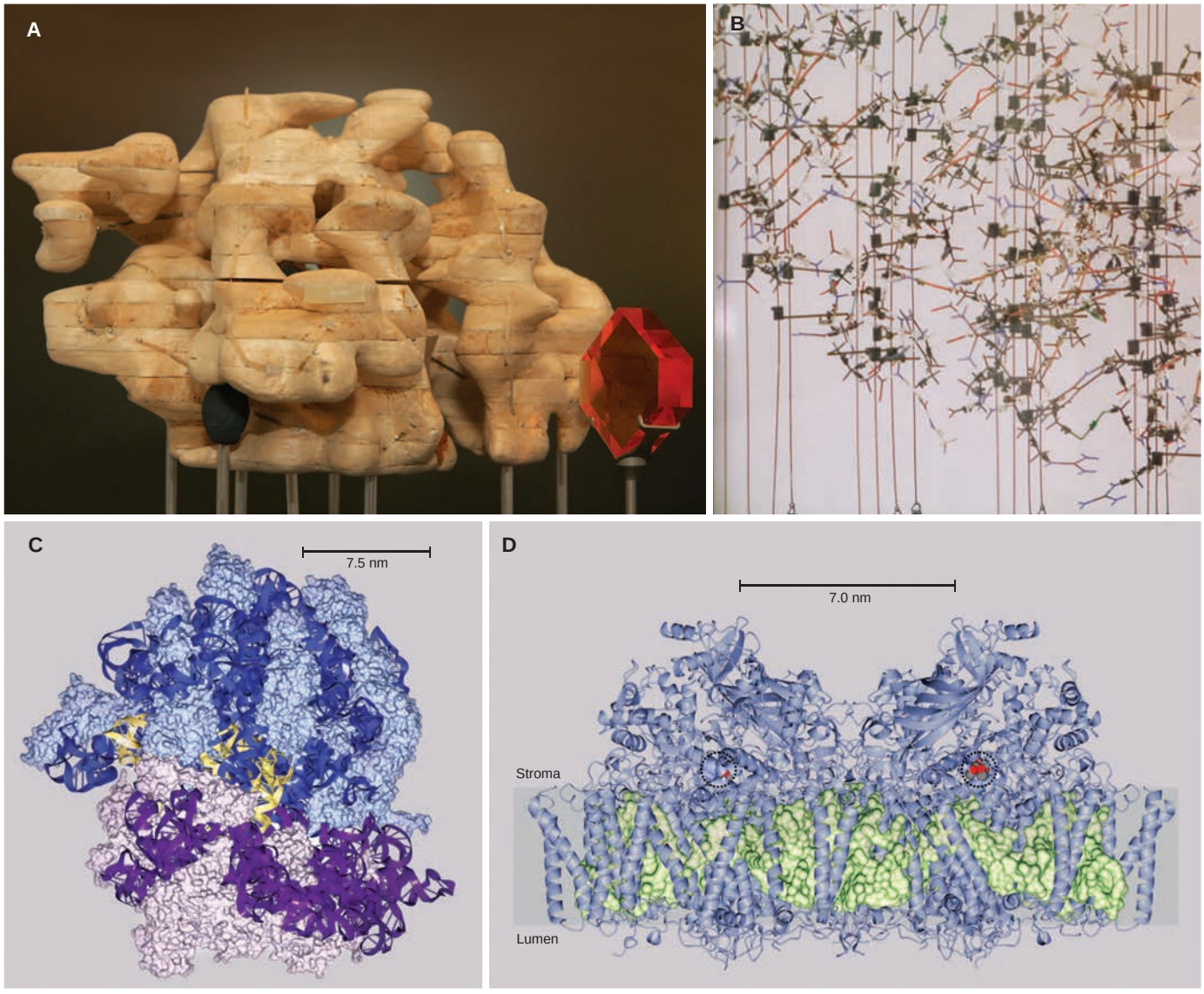

Fig. 1. Visualization of macromolecular structures. (**A**) Balsa wood model of myoglobin at 5 Å resolution (*45*) and a model of a monoclinic crystal, made by H. Scouloudi, 1969. (**B**) Wire model of lysozyme structure (*39*). Model constructed by W. Browne and M. Pickford circa 1965. Refurbished by A. Todd and Unicol Engineering of Headington, Oxford, UK. Blue, nitrogen; red, oxygen; black, carbon; yellow, sulfur; and gray, hydrogen bonds. (**C**) Ribosome 70*S* particle at 3.5 Å resolution (*46*). 30*S* subunit and tRNA, PDB entry 2wdk; 50*S* subunit, PDB entry 2wdl. The 30*S* subunit is shown in purple (pale for protein, dark for RNA) and the 50*S* subunit in blue (pale for protein, dark for RNA). The tRNA is in gold. Figure made with CCP4mg (*47*). (**D**) Photosystem II at 1.9 Å resolution. PDB entry 3arc (*48*). The protein is shown in blue and the chlorophylls in green. The oxygen-evolving cluster is depicted as spheres and highlighted by dotted circles, and the membrane bilayer is indicated by a shaded box. Figure made with CCP4mg.

critically depends on crystalline order, which usually deteriorates if the crystals are allowed to dehydrate. Many of the technical challenges in the field arise from this property of protein crystals.

Crystallographic macromolecular structures are time and space averages over the many millions of macromolecules within the crystal. A "large" protein crystal is typically smaller than 100 μm in all three dimensions. For an average-sized 5-nm-diameter globular protein, such crystals would contain ~10^{13} molecules. The dynamical behavior of the molecules within a crystal allows only a limited sampling of the conformational space of the protein because the crystallization conditions bias the behavior. Better information on dynamical properties is required to fully understand protein-protein interactions and pathways. Techniques to address this issue are being explored with the aid of newly available technology, and current approaches are described elsewhere in this issue (*2*).

For the past 20 years, over 95% of macromolecular structures have been determined from crystals held at cryotemperatures (~100 K) because the rate of radiation-induced damage is lower by a factor of ~70 compared with room temperature (*3*). Although 100 K is far from physiologically relevant temperatures, it is clear from structural studies of the same proteins at different temperatures that the overall fold of the alpha-carbon amino acid chain is temperature independent. More ordered water molecules can be located in structures determined at cryotemperatures, and alternative conformations of side chains tend to be better defined. This is because the dynamic disorder in the protein is "frozen out" and the observed substate populations reveal only the static disorder. Because these detailed observations are not necessarily physiological relevant, ideally structures would also be determined at room temperature if this could be conveniently expedited.

Currently, some promising new developments in macromolecular crystallography are unfolding. Future growth areas summarized below are membrane protein crystallography, and room-temperature data collection both at synchrotrons and at the recently introduced x-ray free-electron lasers (XFELs).

The Pipeline

The deployment of new technology and methodology is continually streamlining the pipeline involved in macromolecular structure solution (Fig. 2) and improving the success rates for challenging cases. However, the major bottleneck remains the growth of diffraction-quality crystals.

Before crystallization can be attempted, sufficient quantities of protein must be purified, usually as recombinant material from bacterial, yeast, insect, or mammalian cells. Expression systems have become high throughput as a result of more rapid and reliable cloning tools and the more widespread use of automation and bioinformatics. These developments permit better-informed and extensive screening of expression vectors, protein sequences, and heterologous host cells (*4*). It can still be a labor-intensive and time-consuming task to optimize the system to produce enough protein for crystallization trials. However, with recent methodological progress, the structures of an increasing number of proteins that were historically viewed as challenging (e.g., membrane proteins, posttranslationally modified proteins, and protein complexes) are now being solved.

An important development has been the use of autotrophic strains for the incorporation of seleno-methionine into recombinant protein, because the selenium allows the structure to be experimentally phased by the multiwavelength anomalous dispersion (MAD) method (*5*).

To maximize the chances that crystals will grow, the protein must be as homogeneous and pure as possible, so it must usually be in a single oligomeric state. Large losses of protein may be experienced during purification, but this step is vital for successful crystallization. Techniques for assessing protein purity have advanced considerably, and a variety of methods are now used, including dynamic light scattering and coupling of size-exclusion chromatography with multiangle laser light scattering. These reveal whether a protein sample is monodispersed and homogeneous, often giving a good indication as to whether it might crystallize.

Although the parameters governing the process of protein crystallization are now better understood through research into crystallogenesis, it is not yet possible to predict the conditions under which a particular protein will crystallize. Thus, the approach is still to coarse-screen a wide range of chemical conditions—such as buffer type, temperature, pH, protein concentration (typically 10 to 20 mg/ml), cocktails of detergents if it is a membrane protein, precipitants (organic solvents, salts, and polymers), presence or absence of divalent cations, and additives—in the hope of obtaining a few hits. Screening on a finer grid that samples around these promising conditions then allows optimization, which may result in diffraction-quality crystals.

Crystallization robots that can routinely dispense low-volume drops (as low as 50 nl protein + 50 nl of precipitant solution) permit thousands of conditions to be coarse-screened. This has greatly increased the likelihood of crystallization conditions being found given limited protein volumes; for instance, with 150 μl of protein, ~1500 trial drops of 100 nl + 100 nl could be tested in slender 96-well plates holding two conditions per well. Larger volume than the minimum 50 nl is usually dispensed, because scaling up crystallization conditions from such small drops can be problematic due to changes in surface-to-volume ratios. The trays are typically kept at a constant temperature (e.g., 4°C or 20°C) in "crystal hotels" equipped with imaging devices that automatically photograph the crystallization drops at regular intervals, and these images can then be scored using automated crystal recognition software. Thus, much of the drudgery has been removed from the search for suitable conditions. The successful development of such automated systems owes much to the investment of resources and time made in structural genomics centers in the early part of this century.

Once a crystal has been obtained, it must usually be manually harvested from its growth drop before

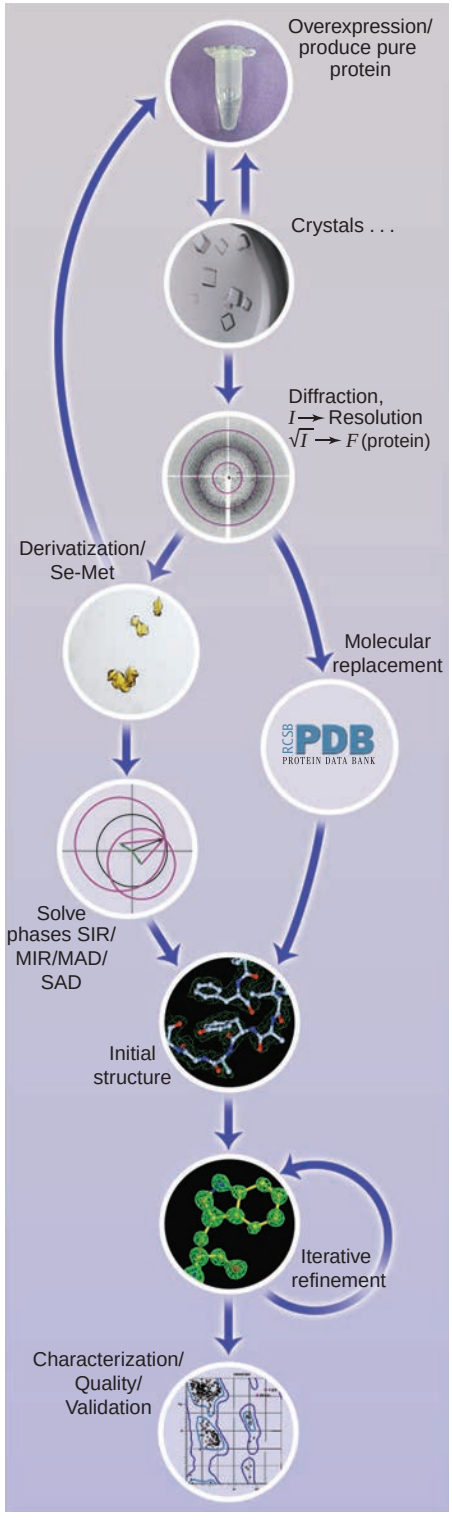

Fig. 2. Diagram showing, from top to bottom, the pipeline for macromolecular structure solution.

being irradiated with x-rays. Successful vitrification (Fig. 3) of the crystal for data collection at cryotemperatures generally requires the presence of cryoprotectants. The flash-cooling of crystals (*6*), held in cryoloops by surface tension, is a step in the macromolecular crystallography pipeline that has so far proved difficult to automate. Commercial cryoloops are available in a range of sizes and made from rayon, microfabricated polyimide film, and etched mylar, some having integral meshes to support fragile crystals or many small crystals simultaneously. Technically, there is a pressing need for automatic crystal harvesting and sample handling methods to overcome this pipeline bottleneck.

The evolution of storage ring sources to the currently available third-generation synchrotron sources (*7*) (Fig. 4) in conjunction with fast and accurate x-ray detectors has revolutionized macromolecular crystallography for the collection of diffraction data. The very high synchrotron source flux densities (photons per s per mm^2) allow weakly diffracting or smaller crystals to be used for structure determination. They provide parallel and stable beams, many of which can be tuned to deliver incident x-ray energies from 6 keV to 20 keV (~2.1 to 0.62 Å), giving access to the absorption edges of a wide range of metals for experimental phasing by the MAD method. Pioneering beamlines suitable for data collection at longer wavelengths (up to 4 Å) are under construction to enable more experimental phasing of structures using the anomalous signal from intrinsic sulfur atoms in proteins. The now robust top-up mode at synchrotron sources, in which the storage ring is continuously fed with electrons, results in stable experimental conditions for long periods of time. Detector technology has moved on apace, driven by the requirement for faster and larger position-sensitive devices. Originally, the field used photographic film and proportional counters, and then position-sensitive multiwire gas-filled detectors, adapted television tubes, imaging plates (reusable film), charge-coupled device detectors, and, most recently, pixel detectors (*8*).

Most synchrotron beamlines are currently equipped with sample-mounting robots that transfer crystals from a liquid nitrogen Dewar to the goniometer into a stream of 100 K nitrogen gas, meanwhile keeping them cryocooled. The increased reliability of these robots has led to remote data collection in which crystals are delivered to the beamline and the researcher controls the beamline hardware remotely. Synchrotron beamline availability is now such that many in-house systems are being decommissioned.

A number of synchrotron beamlines are now providing particular special facilities, such as microfocus beams (diameters down to 1 μm). With the necessary supporting software, these beams can be used to map the diffraction properties of a crystal so that the best place for data collection can be selected. To minimize background and maximize the signal-to-noise ratio, the beam and crystal size should be matched. Thus, these microbeams are ideal for use with microcrystals, where many crystals can be mounted on one loop and then individually irradiated.

Additional instruments have been made available to augment the information that can be obtained from crystals through simultaneous data collection using complementary techniques. For example, most synchrotrons now have a beamline onto which a microspectrophotometer can be mounted, which can provide valuable data on redox protein states and radical formation during x-ray irradiation (*9*). Another useful new addition is a device to carry out on-line controlled dehydration of protein crystals (*10*), because in some cases this technique can improve the diffraction quality in a reproducible way. For instance, F1 adenosine triphosphatase crystals were improved from 6.0 Å to 3.84 Å resolution by dehydration (*10*).

Automated data reduction pipelines are now widely available at most beamlines, and these allow on-line evaluation of the results so that more data can be collected immediately if necessary, substantially improving the outcomes of the experiment. However, even for cryocooled crystals, the age-old problem of radiation damage remains an issue and can result in failed structure solution due to the degradation of diffraction quality and the onset of specific structural damage (*11*) before enough data have been obtained. Research is ongoing to understand the variables involved and to seek mitigation strategies (*12*). The extent of damage at cryotemperatures is proportional to the absorbed dose, and an experimental dose limit of 30 Mgy, beyond which structural information may become compromised, has been determined (*13*). Software (Raddose-3D) is available to model 3D dose profiles for a range of experimental strategies (standard, helical, and translational). These simulations can be used to plan experiments that result in more homogeneous dose distributions, reducing the extent of differential radiation damage across the sample and improving data quality (*14*).

A number of streamlined packages are available to analyze the diffraction data and to reduce them to a unique set of reflections so that structure solution can commence. Concomitant with the developments in hardware and the automation of data collection, computational tools for structure solution have seen huge progress over the past decade. Crystallographic software, such as that distributed by Collaborative Computational Project Number 4 (CCP4) (*15*) and PHENIX (*16*), can now solve many structures without human intervention, from data reduction through phasing and electron density map calculation, map interpretation (model building), structure refinement (completion), and deposition in the Protein Data Bank (PDB). For the cases in which automated solution is still not possible, the software is better able to analyze the pathologies causing it to fail and to guide the crystallographer to a manual solution. Molecular replacement can now succeed with very distant models or even secondary structure elements, as implemented in Phaser (*17*) and Arcimboldo (*18*). Experimental phasing can now succeed with very weak anomalous signals due to progress in phasing software [e.g., the SHELX suite (*19*)] and improved methods to enhance the anomalous signal when combining data collected from a large number of different crystals [e.g., (*20*)].

After an initial model is obtained, the structure must be refined to optimally match the model to the electron density. This process is fast and has a wide radius of convergence—for example, in Phenix.refine (*16*) and Refmac (*21*). Software for automatically building atomic models into electron density maps is increasingly more robust, and for manual building, programs such as Coot (*22*) tremendously aid the iterative process of model refinement and rebuilding. The graphical capability now available allows macromolecules to be represented much more speedily, cheaply, and conveniently than with balsa wood and wire models (Fig. 1, A and B). For the last step in the pipeline, convenient new tools are also available for the validation of the geometry and quality of structures before submission of atomic coordinates to the PDB (*23*).

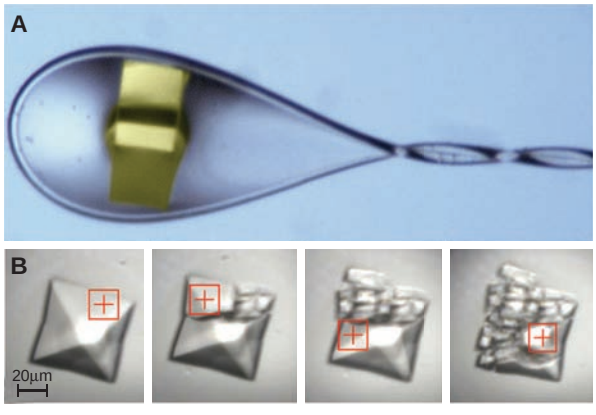

Fig. 3. Macromolecular crystals ready for data collection. (A) Cryocooled 0.5-mm-sized crystal of *Salmonella typhimurium* neuraminidase in a 20-μm-thick rayon fiber cryoloop held in a 100 K nitrogen gas stream. The transparent film of solid cryobuffer supporting the crystal indicates that no crystalline ice has formed that could interfere with the crystal diffraction pattern. **(B)** In situ data collection from bovine enterovirus crystals; despite the rapid and dramatic disruption of the crystal lattice, small amounts of high-quality data can be collected in a serial manner until a complete data set is obtained (*30*). Reproduced by permission of the International Union of Crystallography (IUCr).

Future Growth Areas

Current growth areas in which macromolecular crystallography is likely to have considerable future impact include membrane protein structure solution, renewed interest in room-temperature structure

determination at synchrotrons, and the possibilities offered by XFEL x-ray sources.

About 30% of the proteins coded by the human genome are membrane proteins. Determining the structure of these represents a major challenge for conventional techniques, because the crystallization step usually relies on controlled dehydration of a solution of protein. Because proteins extracted from the membrane are by their very nature insoluble in aqueous systems, new methods have to be employed to obtain crystals; the proteins must normally be solubilized in detergents, both throughout purification from cell lysates and during crystallization. This greatly increases the number of variable crystallization parameters to be explored and makes the search for suitable conditions both time-consuming and expensive. The addition of detergents is prone to destabilize the protein, and much trial and error is required for successful outcomes. As a result, out of 97,362 protein structures (as at 28 January 2014) deposited in the PDB, there are only 1394 membrane protein structures (*24*), although the number is increasing rapidly. In part this is due to the development and success of a new crystal-growing technology: the "in meso" method, which makes use of lipidic mesophases and is also referred to as the lipid cubic phase (LCP) method. This uses monoolein, which has a well-characterized phase diagram of composition (water/lipid) against temperature (*25*). Crystallization robots to dispense LCP are now available, and they substantially simplify and accelerate the setting up of screens. However, safe removal of crystals from LCP material requires skill and patience on the part of the experimenter, so this stage is ripe for further innovation. On contact with air, the LCP can swiftly dehydrate unless additional crystallization solution is added, and it also becomes opaque and birefringent, making it hard to locate and to harvest the crystals. Once in a cryoloop and flash-cooled (no added cryoprotectant is needed) for cryodata collection, the LCP again often becomes opaque, and any crystals within it become invisible. The automated grid scans of the x-ray beam over the loop area to detect crystal diffraction above have alleviated this problem, and work to image such crystals by x-ray microradiography and microtomography is ongoing (*26*).

Membrane protein crystals grown in cubic and sponge phases have yielded data revealing, for example, the structural basis for the counter-transport mechanism of a H^+/Ca^{2+} exchanger (*27*) and the structure of the ß2 adrenergic receptor–G protein–active complex (*28*), a GPCR in association with its cognate G protein. Correct functioning of GPCRs is vital for our senses of smell, taste, and sight and is also involved in almost all signaling processes, including cellular responses to neurotransmitters and hormones. Because roughly half of all modern drug targets are GPCRs, their structural elucidation is one of the major highlights of recent research.

The ability to crystallize membrane proteins in a membrane-like environment such as LCP opens the possibility of gaining more biologically relevant information on protein-lipid interactions. Such interactions help regulate subcellular localization and determine the activities of transmembrane proteins, yielding, for instance, insight into the function of the receptor tyrosine kinase family. These proteins are implicated in the progression of many types of cancer, as well as being vital regulators of normal processes in the cell (*29*).

In the search for suitable crystallization conditions for membrane proteins, it is often highly instructive to test the diffraction properties of putative crystals obtained from a coarse crystallization screen. This necessity has prompted beamline scientists at a number of synchrotrons to adapt conventional goniometers so that entire 96-well crystallization plates can be mounted in the x-ray beam and translated to enable irradiation of individual wells containing putative crystals. In some cases, a limited rotation capability has also been incorporated into the beamline hardware and software, so that complete ensemble data sets constituted of images from many crystals can now be collected and can result in successful structure solution (*30*), without the necessity for any post-growth handling of crystals. Figure 3 shows a crystal of bovine enterovirus at room temperature in a

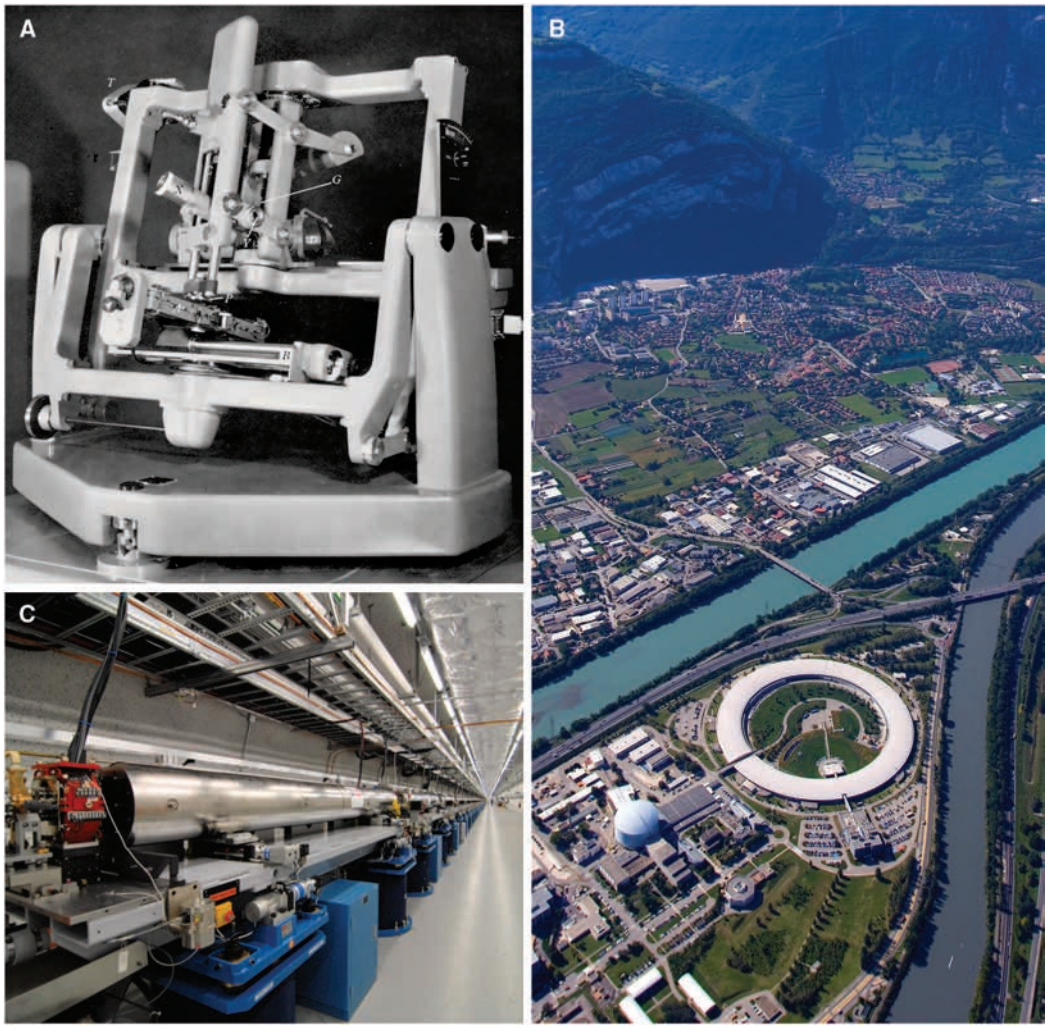

Fig. 4. Progression of hardware for macromolecular crystallography experiments. (**A**) A Hilger-Watts linear diffractometer as used to collect the data used to solve the structure of lysozyme in 1965 (*49*). (**B**) The first third-generation synchrotron x-ray source: the European Synchrotron Research Facility (ESRF), Grenoble, France. Photo courtesy of ESRF/Morel. (**C**) Part of an XFEL: a 132-m-long undulator at the Linear Coherent Light Source, Stanford, CA, USA. [Photo courtesy of SLAC National Accelerator Laboratory, Archives and History Office]

crystallization tray being consecutively irradiated for 0.5 s at four different positions by translating the tray before radiation damage effects cause the disintegration of the recently irradiated part. The success of this strategy relies heavily on the high speed of data collection and on the advent of extremely fast pixel array x-ray detectors (PADs) (*31*). These are replacing the charge-coupled device detectors that have been the macromolecular crystallography workhorses for the past 10 years.

Currently, the biggest PAD is 425 by 435 mm^2 and has a readout of 0.995 ms, a maximum frame rate of 100 per second, and 6 million pixels. The PAD readout times are so fast that they have resulted in a paradigm shift in the way the diffraction experiment is carried out, with shutterless data collection becoming the norm: It is now unnecessary to oscillate the crystal over a limited angular range (~0.1 to 1°) and then close the shutter during detector readout. This change in experimental approach combined with the high PAD frame rates dramatically increases the rate at which data can be collected, while concomitantly reducing demands on beamline components such as x-ray shutters.

Experiments using a high-speed PAD have demonstrated that it may be possible to collect data at room temperature so quickly that the catastrophic effects shown on Fig. 3B can at least partially be "outrun" (*32*). There was already anecdotal evidence from early macromolecular crystallography synchrotron experiments 30 years ago that room-temperature crystals lasted much longer than had been expected, and during the past 5 years there has been some debate as to the existence of a room-temperature dose-rate effect on radiation damage progression. It would be most instructive to understand the details of the radiation chemistry pathways in room-temperature protein crystals during x-ray irradiation, so that the application of recent technological developments could be optimized.

In conjunction with the in situ tray irradiation described above, the opportunity to collect more room-temperature diffraction data by collecting it faster has opened up the potential for protein structures to be determined with no postgrowth handling being necessary. This is particularly pertinent for virus crystals for which biological containment requirements complicate traditional data collection methods, but it is also important for samples that prove difficult to handle or manipulate and for those that cannot be cryocooled without serious degradation of their diffraction properties.

Hardware developments for macromolecular crystallography have not been confined to the improvement in the size and accuracy of x-ray detectors. Since the early days of sealed-tube x-ray sources, crystallographers have exploited the latest technical advances to obtain brighter beams. The huge increase in source brilliance (B) (measured in units of photons per second per mm^2 per millisteradian per 0.1% bandwidth, here called U) available today has been achieved through steady progress that has encompassed rotating anode x-ray generators with magnetic liquid rotary vacuum seals ($B > 10^7$ U), focusing optics fabricated from alternating graded layers of high and low–atomic number elements ($B > 10^8$ U), synchrotron-fed electron storage rings equipped with bending magnets ($B > 10^{10}$ U), wigglers ($B > 10^{11}$ U), and then ultimately in-vacuum undulators ($B > 10^{12}$ U), and finally the recent advent of XFELs at Stanford [Linear Coherent Light Source (LCLS)] (Fig. 4), SPring8 Angstrom Compact Electron-Laser (SACLA), and Deutsches Elektronen-Synchrotron (DESY) [Free Electron Laser Hamburg (FLASH)]. For example, the macromolecular crystallography CXI (coherent x-ray imaging) beamline at the LCLS is typically operated at 10 to 120 Hz, with x-ray pulses of around 10^{12} photons in a 10-μm focus, which can be tuned from 70 to 300 fs at energies of 4 to 10 keV ($B_{peak} > 10^{33}$ U; $B_{average} > 10^{21}$ U).

Serial femtosecond crystallography (SFX) is a technique in which protein nanocrystals suspended in a liquid jet are streamed using a surrounding gas jacket (*33*) perpendicular to the beam direction so that the x-ray pulses hit them to produce diffraction stills. These patterns are recorded on special PAD detectors (*34*). Typically, hundreds of thousands of images are collected, a small fraction of which show a diffraction pattern, and a small percentage of these are suitable for structure solution. The collection of one still image per nanocrystal presents a major challenge for available diffraction analysis software. In an ongoing effort, new methods (e.g., Monte Carlo integration) are being employed to extract useful information from the many terabytes of data collected during every XFEL run.

Notable SFX results so far include the structures of Cathepsin B (*35*) and photosystem I (*36*), both determined by the molecular replacement method. In another highlight, a combined spectroscopic and crystallographic study gave insights into the workings of Photosystem II (*37*), a large complex of transmembrane molecules (Fig. 1D), vital to photosynthesis and thus to aerobic life. In late 2013, a proof of principle de nuovo structure determination of soaked lysozyme nanocrystals (<1 by 1 by 2 μm^3 in volume) was achieved using the anomalous signal from a gadolinium cluster derivative (*38*), a serendipitous link with the beginnings of macromolecular crystallography (Fig. 1B), and technologically a far cry from the original lysozyme structure determination using a Hilger-Watts linear diffractometer (*39*) (Fig. 4). Thus, XFEL sources promise to provide a way in which new structures can be experimentally phased for proteins and protein complexes for which only nanocrystals can be grown.

Although the full potential of XFEL sources for structural biology has yet to be realized, they are starting to fulfill their promise of overcoming the problem of radiation damage by allowing "diffraction before destruction" during the diffraction experiment. Coupled with innovative technical developments to overcome the considerable challenges posed by the need to deliver crystals, including those grown in LCP into the path of the in vacuo XFEL sample chambers, they are also providing inspiration for future experiments to advance biological discovery. In particular, given enough brightness, the XFEL potentially enables the imaging of single molecules. This capability has been shown for the 400-nm-diameter mimivirus at a resolution of 32 nm (*40*).

However, due to the very limited availability of XFEL beamtime, the more involved data processing pathway, and the large amount of crystalline material required, the vast majority of data collection for structural biology for the foreseeable future will be carried out at third-generation synchrotron sources, at which the pipelines for data analysis and structure solution are now well established.

The three topics summarized by no means exhaust the current activity in advancing the field of macromolecular x-ray crystallography. Other interesting areas include new methods for analyzing the electron density obtained from fragment screening experiments to aid drug discovery (*41*) and new synergy between purely computational approaches to structure prediction (e.g., Rosetta) and refinement of structures from diffraction data (*42*), which opens new avenues to address ever more challenging problems.

Conclusions

From its beginnings in 1913 with the determination of the structure of rock salt (two atoms) (*43*), x-ray crystallography has seen many developments that have moved it into center stage as an essential discipline contributing to a broad portfolio of scientific areas. It now has the capability to define the structures of assemblies of biological molecules with as many as 300,000 nonhydrogen atoms. Since its inception, methodological developments have driven the biological insights gained from crystallography, and they will continue to do so for the foreseeable future.

Macromolecular crystallographers have organized one of the earliest examples of a repository of "big data" that is accessible worldwide and is free for academic use. This is the Protein Data Bank, into which all 3D coordinates and the corresponding structure factors (the Fs in Fig. 2), must be deposited before publication. The field has also blazed a trail in making extensive use of statistical validation tools such as the free R value (*44*) and in providing well-tested, thoroughly documented, and continuously supported free software necessary for structure solution (*15*, *16*). In these respects, macromolecular crystallography is a vanguard for other research areas to follow.

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REVIEW

Femtosecond Crystallography with Ultrabright Electrons and X-rays: Capturing Chemistry in Action

R. J. Dwayne Miller[1,2]

With the recent advances in ultrabright electron and x-ray sources, it is now possible to extend crystallography to the femtosecond time domain to literally light up atomic motions involved in the primary processes governing structural transitions. This review chronicles the development of brighter and brighter electron and x-ray sources that have enabled atomic resolution to structural dynamics for increasingly complex systems. The primary focus is on achieving sufficient brightness using pump-probe protocols to resolve the far-from-equilibrium motions directing chemical processes that in general lead to irreversible changes in samples. Given the central importance of structural transitions to conceptualizing chemistry, this emerging field has the potential to significantly improve our understanding of chemistry and its connection to driving biological processes.

Chemistry has long been appreciated to be a race against time. One wants to create conditions to drive the desired chemistry faster than other possible reaction routes. To this objective, we have been left to imagine the relative atomic motions that lead the system through an activation or energy barrier to convert to new chemical species. This conceptualization of chemistry represents a classic thought experiment that provides the unifying language connecting the different disciplines in chemistry as well as provides the conceptual bridge between biology and chemistry. The challenge is to depict transition-state structures that are taken to be energetically at the halfway point along an assumed reaction coordinate connecting reactant and product states. This exercise is a useful pedagogical tool because it emphasizes the connection between the structure at critical transition points and barrier heights. We need this structural connection in order to properly think about means to control barrier heights and thereby the chemistry (and biology) of interest. This practice can be justified for few atom systems but is questionable for most systems of chemical interest. For a molecule of N atoms, there are on the order of $3N$ degrees of freedom or dimensions to the problem to track all possible nuclear configurations. Imagine trying to map a surface with hundreds of dimensions to give you all the routes interconnecting different possible stability points. It would be extremely difficult to find general features for trekking between one stable valley, or structure, to another. Here, one has to marvel at chemistry. Within the classic description of transition-state processes, each molecule would have a distinct many-body potential energy surface, with distinct modes reflecting the different degrees of freedom needed to describe the nuclear fluctuations. Each different molecule should be a new adventure; yet, chemistry involves widely applicable reaction mechanisms—that is, transferable concepts.

The problem to date is that we have been unable to observe the key modes involved in directing chemistry. We have a very detailed understanding of equilibrium fluctuations of molecular systems based on vibrational spectroscopy as well as a host of other experimental and theoretical methods. However, until recently there has been no direct means to observe the primary atomic motions involved in structural transitions. With the recent advances in ultrabright electron and x-ray sources, it is now possible to light up the atomic motions (via diffraction) on the prerequisite time scale to observe the key modes governing chemistry (*1*).

"Making Molecular Movies"

To get some appreciation of the experimental challenges, consider trying to build a camera to capture atomic motions on the fly, to make a "molecular movie." What is the shutter speed required to follow chemically relevant atomic motions? If we use the case of bond breaking, the time scale involved is the time it takes two atoms to move far enough apart so that the interatomic potential is no longer binding within k_BT (where k_B is the Boltzmann constant and T is the temperature).

[1]Atomically Resolved Dynamics Division, The Max Planck Institute for the Structure and Dynamics of Matter, The Hamburg Centre for Ultrafast Imaging, Luruper Chaussee 149, Hamburg 22761, Germany. [2]Departments of Chemistry and Physics, University of Toronto, 80 St. George Street, Toronto M5S 1H6, Canada.

If we take as upper limits a bond displacement of 1 Å (10^{-8} cm) and two atoms moving apart at the speed of sound (10^5 cm/s) along this coordinate, the time scale for reaching the point of no return or bond breaking would be on the order of 10^{-13} s, or 100 fs (*2*). This time scale should be familiar. It is associated with the typical thermal sampling time from Arrenhius theory for unimolecular reactions. There are faster nuclear motions. For example, one of the highest-frequency motions is that of the OH stretch in liquid water, with a period of 10 fs. However, this motion involves very small displacements, < 0.1 Å, which is well-approximated as motion within a harmonic potential. Chemistry, as discussed above, involves far-from-equilibrium motions (angstrom scale) that involve highly anharmonic potential energy surfaces. It is these motions that are key to understanding chemistry. The time scales of evolution along reaction coordinates depends on the specific modes involved and nature of the potential energy surface at the barrier-crossing region. For the present purposes, we can use 100 fs as the canonical shutter speed needed to capture the primary motions involved in directing chemistry.

Now, consider the lighting requirements. If we wish to capture atomic positions, we need a source with carrier wavelengths comparable with or smaller than the interatomic spacing. This requirement restricts the source to either hard x-rays or high-energy electrons. Keeping within this camera analogy, image quality always depends on having sufficient lighting. The faster the shutter speed, the brighter the source needs to be to keep the image quality for a given detection method. In terms of the machine physics, it was the source brightness that was the biggest technical challenge, as will be elaborated on below.

Last, consider the film requirements for a molecular movie camera. The film providing the image is the sample. The highest spatial resolution is generally attained by using single crystals and resolving atomic structure through diffraction or reciprocal space imaging. The exposure time of the film or sample is limited by x-ray and electron-induced damage and even more greatly limited by the damage introduced by the excitation pulses needed to trigger the chemistry of interest (vide infra). The sample is always the limiting factor in crystallography. However, the demands on acquiring sufficient high-quality samples for diffraction reaches a new scale in time-resolved measurements. Ideally, one would like on the order of 100 time points for sufficient dynamic range. If each observation damages the sample under the required sampling conditions (laser excitation), the sample requirements for time-resolved crystallography become enormous relative to conventional crystallography. This challenge has been met through ingenious schemes by using aerosol/liquid jet injectors (*3*) and self-assembling crystallography chips (*4*, *5*) capable of providing thousands to millions of samples or frames for making molecular movies. Nevertheless, as in all crystallography it is the sample that is most limiting with respect to resolution.

One has to also consider the background problem or image contrast within this analogy. The interconversion of matter from one form to another is a rare event involving nuclear fluctuations over a barrier separating two or more stable forms of arranging the constituent atoms. For even relatively small barriers (<0.5 eV), at any given instant there is less than 1 in 10^8 molecules within an ensemble undergoing a thermally accessed barrier crossing. How could you ever discern the reactive motions from background?

One possibility is to trigger chemical processes by using perturbative methods. This methodology has been advanced to the limit of the fundamental time scales of chemical processes (*6*). It is now fairly routine to access the relevant time domain with commercially available femtosecond laser systems, with even the attosecond (10^{-18} s) time domain within reach (*7*). Accessing such time scales does not rely on a fast detector but rather on stroboscopic methods in which an excitation pulse perturbs the system, and at a well-defined time delay, the system is probed. In this manner, the recording can be made with a slow detector in which each time delay represents one frame in the movie of the dynamical variables being probed. The time resolution is determined not by the detector but rather by the duration of the excitation and probe pulses. The phototrigger for the structure changes involves exciting the system from an equilibrium distribution to a new point on the many-body potential energy surface that leads to the chemistry of interest, as shown schematically in Fig. 1. One typically needs quantum yields of at least 10% to extract the nuclear motions involved in the chemistry from competing relaxation channels. At this level of perturbation, the system is generally not fully reversible, and the sample needs to be exchanged between the next excitation-probe sequence. For conventional studies of static structures using x-rays, it is usually the x-ray probe that causes accumulated sample damage. For femtosecond time-resolved studies, it is the excitation pulse that leads to sample damage. It is almost by definition: If one is interested in chemistry, the very act of triggering a chemical reaction (or other structural transitions) involving thermally stable product states will lead to an irreversible change. It is the irreversible nature of the system response that imposes such enormous requirements on source brightness and makes the problem so challenging. The consequences of this seemingly trivial technical detail need to be fully appreciated. As a generalization, it is not good enough to have simultaneous subatomic resolution and femtosecond time resolution. Within the restricted sample constraints (limited "film footage"), the problem requires sufficient source brightness to achieve femtosecond time-resolved structural dynamics near-single-shot conditions. This level of source brightness has been achieved by using new concepts for generating femtosecond electron or x-ray pulses to serve as the stroboscopic probe. The source brightness is now sufficient to light up atomic motions via diffraction on the primary time scales dictating structural transitions (*2*, *8*, *9*).

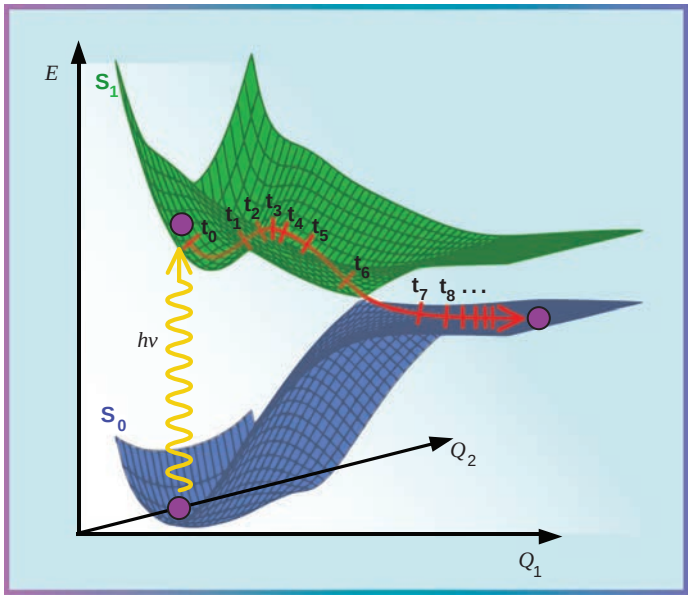

Fig. 1. Phototriggered chemistry. The basic requirement for observing far-from-equilibrium reaction modes is shown schematically. Optical excitation with femtosecond laser pulses prepares the system under barrierless conditions on an excited-state surface ($S_0 \rightarrow S_1$ transition). Even for complex molecular systems, the motions distill down to a few modes that are most strongly coupled to the reaction coordinate. Here, two heavy reduced modes are shown that are representative of an isomerization process (such as an initial step of vision) involving a bond elongation or bond weakening (Q1) and torsional motion (Q2). The time course is indicated as equal time steps in tracking the atomic motions to give some feel of how the ensuing motion of the atoms move in response to the potential energy gradients or reaction forces [adapted from (*79*)].

Evolution in Time-Resolved Crystallography

The first atomically resolved static structures were based on x-ray diffraction that date back to the days of Bragg and Laue (*10*, *11*). Electrons for the most part have been used for real space imaging, although a number of important protein structures have been solved with electrons at various

resolution limits (*12*). The mean free path (coherent interaction length) depends on the scattering cross section and is dependent on composition. For low Z materials such as organics, the electron mean free path is on the order of 100 nm, depending on electron energy. This short coherent sampling depth limits the crystals that can be studied, although electrons are well suited for protein nanocrystallography and tomographic methods for structure determination (*13*). The much better match between the mean free path of x-rays and typical dimensions of high-quality crystals have made x-rays the primary source for high-resolution structure studies that use diffraction. For time-resolved studies, the x-ray–probed volume of interest is no longer defined by the crystal dimensions but by the excitation process. For the femtosecond time resolution needed to capture atomic motions, the peak power of the excitation pulses limits the samples' thickness or excited volume to micrometer dimensions to avoid excessive (terawatts per square centimeter) peak power artifacts, such as multiphoton ionization of the samples (*1*). There are additional considerations with electron probes to avoid blurring from multiple electron scattering. There are standard methods for sample preparation developed for electron microscopy that enable preparation of sub-micrometer samples to meet both conditions. The main difference between electron and x-ray sources reduces to spatial coherence and information content per scattering event. There are also substantial differences in infrastructure requirements as well as source stability that factor into the choice of source for a given experiment.

Electron Source Development

The major challenge in increasing the brightness of electron sources is the inherent coulomb repulsion between electrons. Brightness here is defined operationally in terms of the contrast in the diffraction observable because the spatial focus and intensity are coupled parameters. The higher the electron bunch density or intensity and the larger the transverse spatial coherence, the brighter the source. One way around coulombic repulsion issues, referred to as space charge effects, is to use very few electrons per pulse with single electron pulses being the ultimate limit to completely remove space charge broadening effects. The first experiments of chemical interest were confined to low brightness sources and gas phase systems (*14*–*18*) for rapid sample exchange. The time resolution was limited to the few-picosecond time scale by transit time differences for the electron and laser excitation pulses through the sample (*19*). The achieved time-resolution limit was perfect for resolving transient intermediate structures but not for determining the exact pathway through a barrier or curve-crossing region in the excited state surface. These first gas-phase experiments were pioneering studies and are still considered impressive accomplishments in low signal detection that have yet to be surpassed.

Time-resolved crystallography provides substantially higher spatial resolution and expands the problem selection to condensed phase systems. The higher spatial resolution arises from having many orders of magnitude more molecules aligned under identical conditions, thus amplifying the diffraction process and increasing the signal-to-noise ratio for structure determination. In principle, single-electron sources could be used for such studies (*20*, *21*). However, one needs to collect on the order of 10^5 to 10^6 diffracted electrons for a reasonable diffraction pattern (*22*), depending on the complexity and size of the unit cell. Statistically, to ensure single-electron pulses this class of measurement would then require finding a system capable of more than 10^6 photocycles or 10^6 samples. Photoinduced chemistry in the solid state generally involves irreversible changes, as discussed above. The photoexcited volume also undergoes very slow thermal cooling of the absorbed photon energy, which further conspires to limit sampling rates to unacceptably long data acquisition times in order to avoid thermal artifacts. There is no real substitute for high brightness sources for this class of experiment. Given the inherent space charge or coulombic repulsion effects associated with electron sources, the brightness requirements seemed to be an intractable problem for electrons.

The first major advance in electron source brightness came from a numerical solution to the coupled equations of motion of some 10,000 electrons in modeling experimental conditions for electron pulse propagation in time-resolved diffraction experiments (*23*). These calculations showed that the space charge broadening was confined primarily to temporal broadening, and that within limits, the transverse spatial coherence could be maintained for atomic structure determination with suitable beam focus. This number of electrons is near the single-shot limit for atomic structure determination of relatively simple unit cells. Most important, the calculations showed that high bunch charge-density electron pulses do not lose space-time correlation and that nonrelativistic electrons naturally develop an extremely linear chirp during pulse broadening. Basically, electrons at the front of the pulse stay at the front, and electrons at the back stay at the back as they experience coulombic pulse broadening effects. Two means of generating electron pulses with sufficient coherence and bunch charge for single-shot imaging with atomic resolution emerged. One solution was to develop extremely compact electron guns to prevent excessive pulse broadening with propagation (*8*, *24*). This solution required some rethinking of high-voltage feedthroughs and electron optics, but it is the simplest and most robust electron source concept for this class of experiments.

The other solution exploits the highly linear chirp and conserved spatial correlation so as to temporally compress the pulses at the sample position. There are a number of dispersive elements for electrons, similar to prisms and gratings for optics, capable of compressing such highly linear chirped pulses with high fidelity. The most elegant solution involves the use of a radio frequency (rf) cavity to compress the pulses on axis for either shorter pulse generation (*25*) or higher brightness on target (*26*). Both the compact and rf gun concepts are capable of between 10^5 and 10^6 electrons, with effective time resolution on the order of 100 fs. The time resolution can be further improved to ~30 fs through rf pulse compression by using time stamping methods to correct for timing jitter of the rf pulse compression technology (*27*). Taking into account the approximately 10^6-higher scattering cross section of electrons relative to x-rays and the need for thin samples, this source technology is comparable in both time resolution and detected particle flux with XFELs (10^{12} x-ray photons/pulse) for femtosecond time-resolved crystallography (vide infra). In this sense, these sources are ultrabright. Further increases in electron source brightness are possible with relativistic electron sources that greatly reduce pulse broadening effects (*28*, *29*), with 10 fs time resolution within reach (*1*, *30*). The main disadvantage of electrons is the more involved sample preparation. The clear advantage of electrons is that this source technology is tabletop and provides a very stable source for achieving high signal-to-noise femtosecond diffraction patterns.

The first grainy pictures with sufficient diffraction orders to resolve atomic motions involved in a structural transition on the prerequisite subpicosecond (10^{-12} s) time scale are shown in Fig. 2A (*8*). These frames catch the simplest possible structural transition—the act of melting—but under rather special boundary conditions involved with strongly driven phase transitions. The particular question being addressed by this work dates back to a long-standing debate in the 1930s concerning the onset of the liquid state (*31*). This issue also has ramifications for understanding the state of matter in other extreme conditions, such as the interior of planets or stars (*32*). To put this question in proper context, consider the melting of a block of ice. We all know ice melts from the surface. We also know that if we direct a blow torch to the ice it will melt faster. This everyday experience is referred to as heterogeneous nucleation. What if you could heat up a material so fast that based on extrapolations of heating rates and melt velocities, you would predict that the material should melt faster than the atoms could move or, more correctly, faster than the speed of sound?

The answer can be gleaned from this data (Fig. 2A) directly, without a high level of analysis. The experiment used a special "blow torch"—in this case, a femtosecond laser system—to achieve heating rates approaching 10^{15} K/s for Al (as opposed to ice in the above analogy). At 500 fs, you can see high-order diffraction rings illustrating that the Al is still in its nascent face-centered cubic (FCC) lattice. At 1.5 ps, you can see these rings become dimmer as the initially photoexcited

electrons lose energy to lattice phonons. The increase in root mean square (RMS) motion of the atoms reduces the lattice coherence and corresponding diffraction as described by temperature-dependent Debye-Waller factors. The most astonishing event happens between 2.5 ps and 3.5 ps. There is an incredibly fast lattice collapse in which bonds are broken and the first coordination number of the FCC lattice goes from 12 to an ensemble average of 10 for the unstructured shell-like structure of a liquid. Once reaching the critical point for this degree of superheating, the whole melting process occurred within 1 ps. This time scale has to be fully appreciated. This is 10 times faster than this process could occur through normal heterogeneous nucleation. Rather than melting from the surface in an "outside-in" fashion (heterogeneous nucleation), the system was melting from the "inside-out" (homogeneous nucleation). This provided an atomic view of homogeneous nucleation that could be used to test the accuracy of atomistic molecular dynamics calculations (*33*). From the real space transform, it was possible to discern that the largest changes involve shear atomic motions, with the collapse of the transverse barrier as part of forming the liquid state.

These observations showed how to control nucleation growth to as few as 10 atoms and avoid cavitation-induced shock waves and thermal damage in laser-driven ablation. On the basis of this new insight, a picosecond infrared laser tuned to the OH stretch of water in tissue was developed for laser surgery, with the correct temporal profile to restrict nucleation growth. This method has now been shown to be capable of cutting tissue without scar tissue formation (*34*).

Subsequent work in the area has primarily focused on photoinduced phase transitions. On the femtosecond time scale of the photoexcitation process, the lattice is effectively frozen. The optical transition to a higher lying electronic state instantaneously changes the electron distribution relative to the time scale of nuclear motions. This provides an opportunity to observe the effects of changes in electron distribution and electron correlation energies on bonding by observing the atomic motions in response to these changes. These studies include strongly driven phase transitions involved in nonthermal melting or electronically driven nucleation effects (*35*, *36*); creating states of warm dense matter with a counterintuitive apparent increase in bond strengths at high excitation levels (*32*); and the observation of highly cooperative, coherent, responses to weak perturbations of strongly correlated electron-lattice systems (*37*). With respect to the latter case, the highest-quality atomic movies of structural transitions have been observed in layered compounds that exhibit interesting two-dimensional effects on electron correlation energies and bonding [(*1*, *37*), movies]. Charge density waves (CDWs) are examples in which a small modulation of the atomic positions in the plane leads collectively to higher overall lattice stability. By photoinducing a change in charge distribution, this delicate balance in forces between intra- and interplane coupling is modified, and the lattice relaxes to a higher symmetry state. The atomic movie of the photoinduced suppression of the CDW modulation in TaS_2 is particularly interesting; one can observe a dramatic effect in which both the suppression and reformation of the CDW occur at the fundamentally fastest possible speeds. This work has been extended to other related systems (*38*, *39*), such as $TaSe_2$ (Fig. 2, B and C), in which one observes similar effects—however, with important differences owing to different inter-plane couplings. When one observes such a collective effect at the atomic level, there is an immediate appreciation of the highly cooperative nature of strongly correlated electron-lattice systems. The visual connection to the many-body effects helps to drive home the operating physics in a single measurement. Effectively, it is a direct observation of the electron-lattice coupling.

From a chemistry perspective, the real power of high–bunch charge and -brightness electron sources has been recently demonstrated by using femtosecond crystallography to follow the photoinduced reaction dynamics of organic systems (*40*, *41*), the

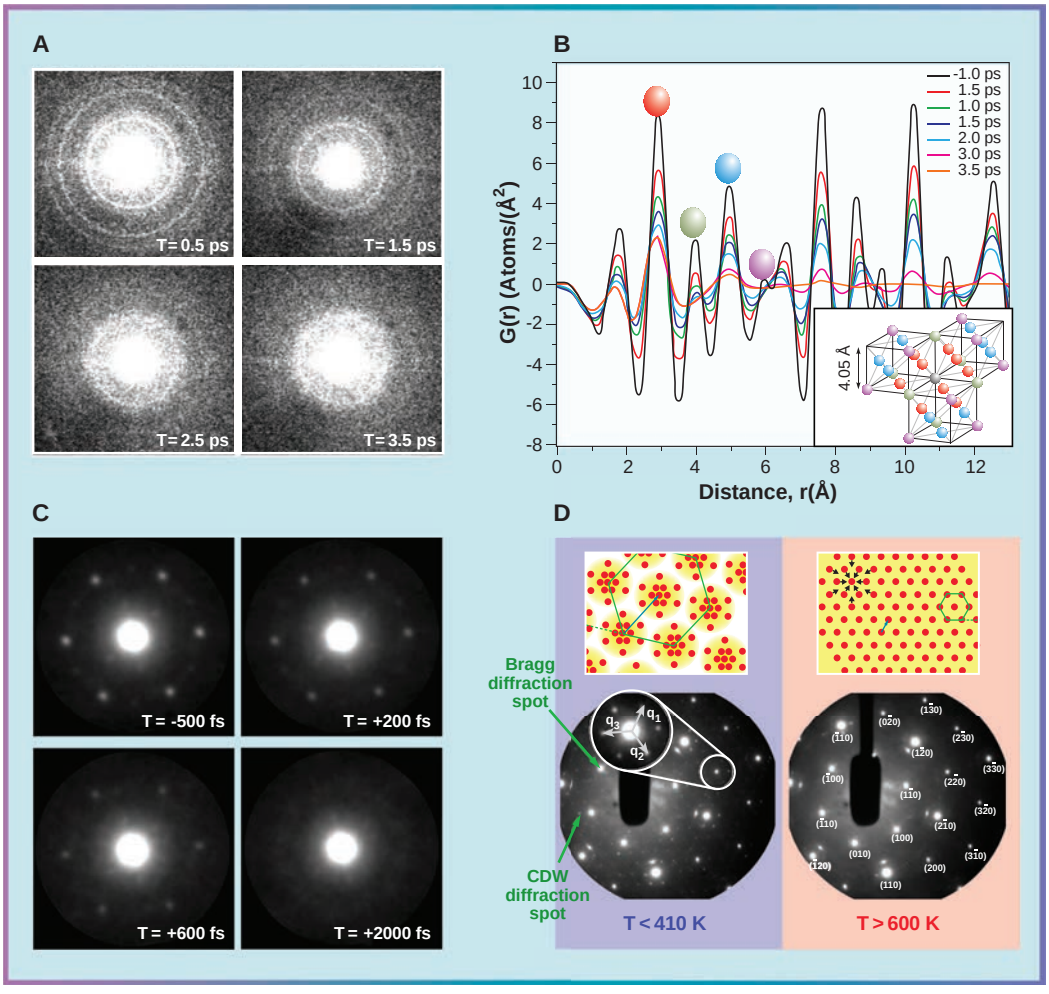

Fig. 2. Evolution in femtosecond electron diffraction. (**A**) The first grainy frames of an atomic movie of the melting of Al under strongly driven conditions. It is possible to see by eye the increased thermal motion leading bond breaking (0.5 to 2.5 ps) and collapse of the FCC lattice to the shell-like structure of a liquid (2.5 and 3.5 ps). (**B**) The real space transform (*8*). (**C**) The dramatic improvement in femtosecond electron diffraction, in which it is possible to directly observe (reciprocal space) the atomic motions involved in the suppression of CDWs in $TaSe_2$ for a single diffraction order out of hundreds to give the structural changes. An expanded view of the two different structures as observed in electron diffraction is shown with a schematic representation of the atomic motions captured on the 100-fs time scale (*38*).

mainstay of chemistry. This class of materials invariably has low thermal conductivities and involve large-amplitude motions as part of the reaction dynamics that greatly limit the sampling rate and number of photocycles. In addition, organic systems have much more complex structures than do the solid-state systems discussed above. The development of high-brightness electron sources was key to opening up this class of study. To gain some appreciation of the quality of the diffraction data that made this possible, compare Fig. 3, a weakly scattering organic system, with Fig. 2A to see the dramatic improvement with source brightness. There were hundreds of diffraction orders that went out to better than 0.4 Å to serve as constraints in the determination of the time-dependent structures. The signal-to-noise ratio of a single diffraction order is comparable with high-quality, integrated, all-optical pump-probe measurements, but with direct connection to structure [(*40*), figure 2, and (*1*), movies].

This improvement in source brightness enabled a dynamic observation of the photoinduced structural changes in the interesting charge-ordered organic system comprising ethylenedioxytetrathiafulvalene (EDO-TTF) and PF_6- counterions, $(EDO\text{-}TTF)_2PF_6$, shown in Fig. 3. This system can be photoswitched from insulating to metallic properties (*40*) by means of a charge-transfer process strongly coupled to nuclear modes, stabilizing the change in charge distribution, as shown schematically in Fig. 3. Inspection of the differences between the insulating and metallic structures shows that the formation of the metallic state involves the flattening of the EDO-TTF moieties. The displacement of a bending mode toward this planar configuration would lead to an increase in the π-π wavefunction overlap between molecules and increased electronic delocalization as part of the onset to metallic properties. Within a conventional transition-state picture, one would naturally expect the bending coordinate to be the dominant mode in this process. However, this simplified line of thinking only works for few atom systems. Considering just the molecules within a single-unit cell, this problem involves over 280 different degrees of freedom or dimensions. However, it was found that all of the diffraction orders could be fit by the displacement of just three reduced modes (Fig. 3C) in which the motion of the heavy PF_6^- counterion appears to be the key mode. In hindsight, this observation is understandable because the photoinduced change in electron distribution will lead to a change in the local field that will exert a force on the counterion. The PF_6^- ion is rather large, and its motion, through steric effects, couples the other modes. The projections along the three reaction coordinates (Fig. 3C) look like shadow projections of one another; the modes are strongly correlated.

One typically uses an approximate frozen slice of a many-body potential to discuss reaction coordinates and get a feel for the forces and types of motion involved in directing the process. However, these results show that the modes are dynamically coupled and that one cannot intuitively guess which modes are involved or the relative degree of coupling. In principle, time-dependent ab initio theory can provide the information on the relative degree of coupling between the different possible motions (*1*). There is a limit. Even the highest level of time-dependent ab initio theoretical methods have to use highly truncated model systems to approximate typical chemical reactions. In this respect, theoretical calculations of reaction coordinates are generally projected along the modes found to be most strongly coupled to the reaction coordinate. Given the level of approximations required in treating electron correlation energies and highly simplified model structures, the observed reduction in dimensionality even within full modal basis calculations might be considered to be a consequence of the truncated moiety used to model the reaction coordinate. We now see that this approach can be experimentally justified for even very complex systems. There is in fact an enormous reduction in dimensionality that again is the key to how chemistry reduces to transferrable concepts in the form of reaction mechanisms.

The other study of an organic system with the necessary space-time resolution to directly observe the correlated atomic motions through barrier-crossing regions was that of the ring-closing reaction of diarylethene (*41*, *42*). This study was distinct in that the process is strictly a chemical reaction and involved bond formation, as opposed to bond breaking. It is generally difficult to set up conditions without major entropic barriers to bond formation. The diarylethylene system was specifically designed to give high quantum yields for cyclization to serve as an efficient photochromic material, capable of undergoing more than 10,000 photon cycling processes (*43*). This system would

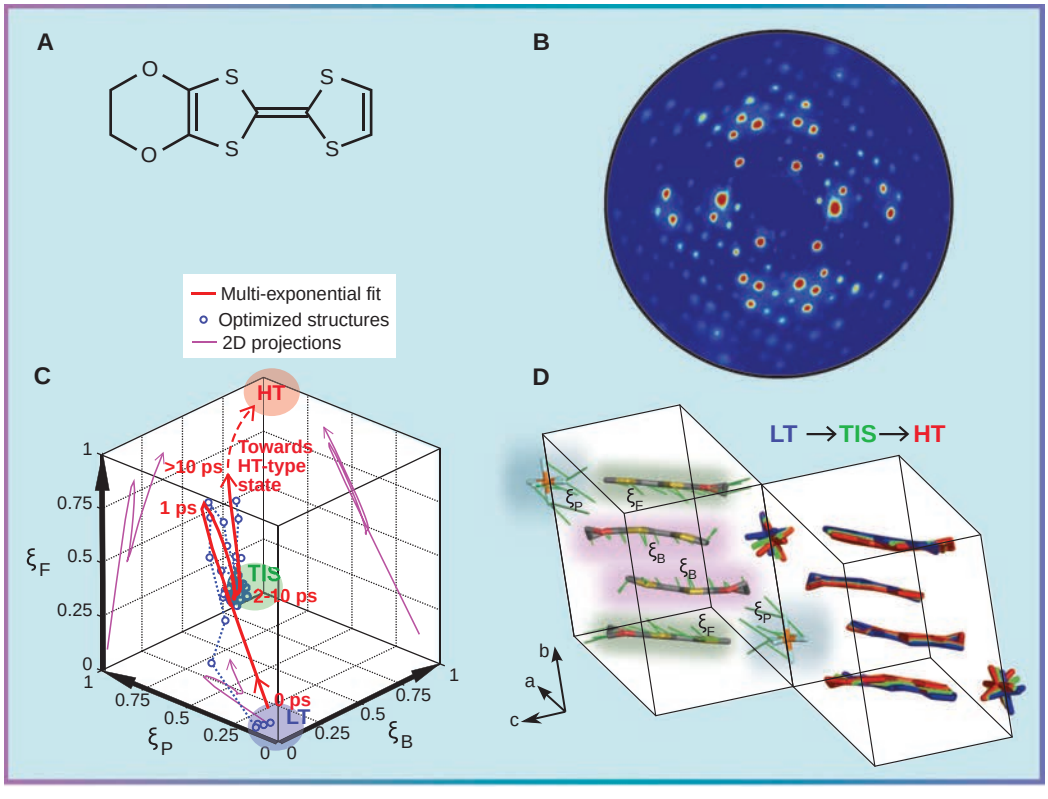

Fig. 3. Reduction in dimensionality. (**A**) Molecular structure of EDO-TTF. (**B**) Representative electron diffraction pattern to illustrate the high quality of diffraction. (**C**) The structural changes can be mapped onto three reduced-reaction coordinates (ξ_P, motion of the PF_6^- counterion; ξ_B, bending coordinate; and ξ_F, sliding motion of the rings) that stabilize the change in charge distribution, leading to electron delocalization and metallic behavior. The projections along these three normalized coordinates are highly correlated, indicating strong coupling between these nominal reaction modes. (**D**) Schematic depiction of the motion along these modes is given to provide a sense of the motions involved, from the insulating structure (LT), to a transient intermediate structure (TIS), to the final metallic-like structure (HT), with direction of motion indicated by the arrows and superposed structures for some sense of animation [from (*40*)].

appear to be an ideal candidate for even single-electron pulse probes. However, this degree of photocycling is only for low fractional excitation. At the excitation levels needed to observe the structural changes above background, even this system is only capable of ~100 photocycles before irreversible changes occur.

This system provides a classic example of a cyclization reaction with conserved stereochemistry. As in the case of $(EDO\text{-}TTF)_2PF_6$, there is an enormous reduction in the nuclear degrees of freedom coupled to the reaction coordinate. A detailed correlation analysis of the femtosecond time-resolved diffraction patterns for the ring-closing reaction found that there is an initial motion occurring around the central bond that involves the whole molecule and brings the labile carbon atoms involved in the bond formation into close proximity (Fig. 4). With these results, it was possible to connect the actual atomic displacements that are best approximated by the lowest frequency, 55 cm^{-1} (*41*, *42*), found in a vibrational mode analysis using density function theory. This is the key mode that directs the system to the seam in the reaction coordinate (Fig. 4A). The question is how does such a spatially delocalized mode lead to the highly localized motions needed to close the ring? Again, there is a surprise. These latter displacements leading to bond formation and ring closing occur on a picosecond time scale involving highly localized rotational motions (*41*). From a time-dependent ab initio calculation, using a truncated model system, these relaxation processes involve additional seams connecting the product and ground state from the excited state. Experimentally, it was possible to cast out a series of localized rotational motions that mix to produce the ring-closed form. The ultrafast nature of this process clearly separates the possible modes that are involved and highlights the importance of sufficient space-time resolution to connect the initial low-frequency mode to the localized rotational coordinates. It is the mixing of modes under the highly anharmonic conditions in the barrier-crossing region that leads to the localized motions and concepts of breaking the weakest bond that chemists have empirically learned to control.

X-ray Source Development

The first time-resolved x-ray diffraction experiments to achieve picosecond to sub-picosecond time resolution were accomplished with laser-based x-ray plasma sources and Thomson scattering (*44*–*48*). The source brightness was initially insufficient to resolve more than a single rocking curve, so it was not possible to connect to structural changes. These initial studies were confined to the study of systems with well-defined, fully reversible atomic motions, such as lattice heating and impulsive excitation of lattice phonons. However, it was possible even within this limited information to provide new insights into the structural dynamics. As a case in point, it was possible to distinguish structural relaxation dynamics for the photoinduced phase transition in VO_2 (*49*), whereas previously it was impossible to separate the electronic and nuclear terms in transient spectra, even without full atomic details.

The next major advance in time-resolved x-ray crystallography came through the exploitation of the time structure of the circulating pulses in third-generation synchrotron light sources. These studies were capable of full atomic resolution, albeit with orders-of-magnitude-lower time resolution as a compromise. The key advance of this work over the laser plasma sources was the ability to use Laue diffraction (broader bandwidth and more information) to maximize the sampled reciprocal space (*50*–*54*). This feature in turn reduced the number of crystal orientations needed to make data acquisition for time-resolved measurements tractable. The initial studies were confined to nominally sub-nanosecond time resolution by the pulse duration of the electron bunch in the ring. There were also substantial challenges in coming up with new analytical methods for extracting atomically resolved transient structures from the unexcited fraction of the crystal (*55*). Long-lived excited states involving intersystem crossing of spin-forbidden transitions and heavy-metal centers were specifically engineered to provide model systems for testing various aspects of quantum calculations for excited states (*56*). The field of time-resolved crystallography quickly evolved, in which it was possible to resolve important details regarding the transient intermediate structures involved in photochemical reactions to bond displacements convolved to changes in spin crossover materials (*57*). These latter studies explored the quantum mechanics of how electrons change spin.

The time resolution of these synchrotron-based sources was improved by a factor of 10^3

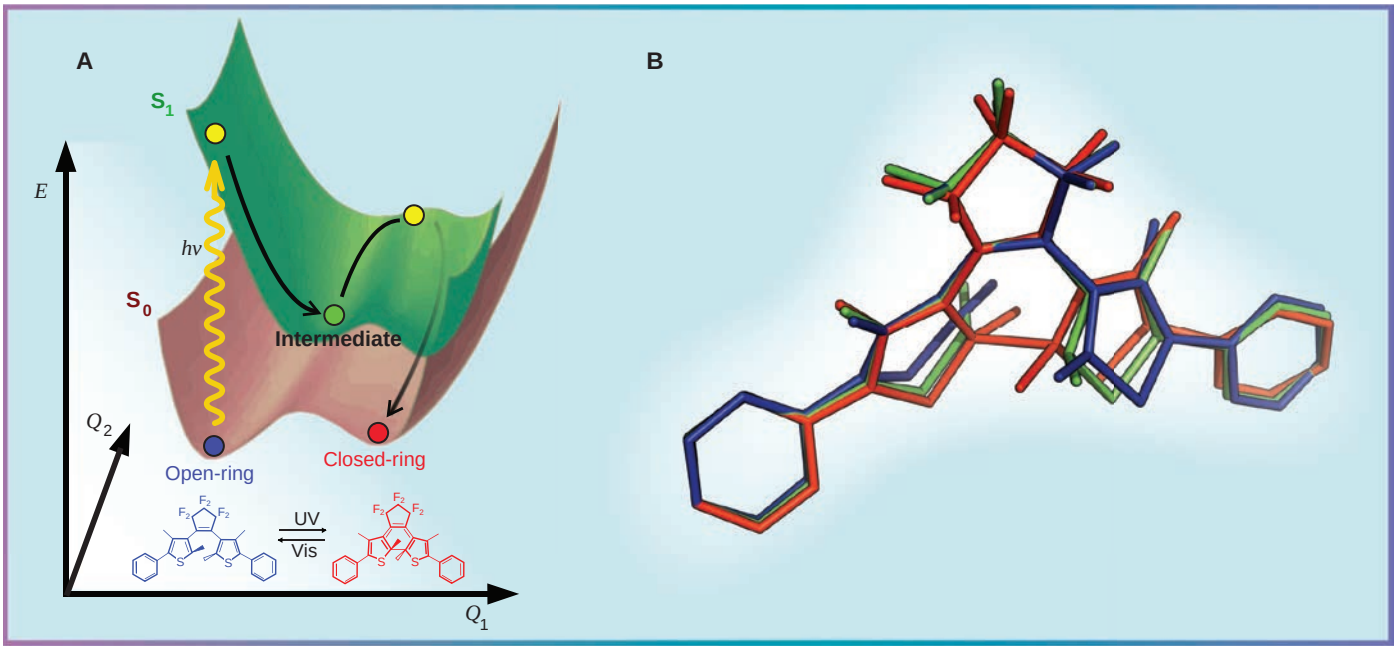

Fig. 4. Observation of the primary motions involved in bond formation and ring cyclization. (**A**) The structure of the specific diarylethene derivative is shown with the photocycle used to follow the ring-closing reaction. The simplified reaction coordinate is shown above for an initial bending motion (Q1) that brings the labile carbon atoms within wavefunction overlap, followed by localized rotation motions (Q2) that close the ring [surface adapted from (*80*)]. (**B**) Animated view of motions recovered from femtosecond electron diffraction studies, from the initial structure (blue), to the distorted bent structure (green), to the final closed-ring structure (red).

to 10^4—to enter the femtosecond domain—by using beam-slicing methods, in which an intense laser interaction with the electron bunch in the ring effectively cuts out a 100-fs x-ray slice from the bunch (*58*). There is a corresponding reduction in x-ray flux by a factor of more than 10^3 with propagation losses. These beam-slicing sources now provide very stable, relatively, broadband sources for femtosecond soft x-ray spectroscopies from which structural information can also be retrieved (*59*, *60*). In terms of hard x-ray diffraction, the source technology is not bright enough to give more than a couple of diffraction orders for simple unit cells. This information is still sufficient to resolve the time scale for structural phase transitions, and electronically driven bond displacements have been tracked on the relevant time scales to give new insights into electronic factors involved in these effects (*61*).

Subsequent to this development, there has been a major advance in laser-based x-ray plasma sources. By going to very thin copper-film targets and using powder diffraction to increase the signal, it has been possible to obtain very-high-quality diffraction patterns with 100-fs time resolution (*62*). The use of powder diffraction to increase the number of diffraction orders was important because it also enabled the use of the background diffraction (unexcited crystal volume) of known structure to serve as a heterodyne source for signal amplification and phase retrieval. Orders-of-magnitude-fewer x-ray photons are then needed to resolve the structural changes. This approach has enabled the inversion of the time-dependent diffraction to very-high-quality electron density maps for the structural changes. These maps have been interpreted in terms of concerted electron-proton transfer in ionic crystals (*62*, *63*), to a very interesting effect involving laser field–driven changes in electron distribution in LiH and $NaBH_4$ (*64*). There is a limitation in that the excitation process involves a nonresonant multiphoton process to uniformly excite the needed x-ray–probed volume for these studies that may lead to multiphoton effects. New advances in the drive laser promise to increase the x-ray flux by two orders of magnitude, which will correspondingly decrease data acquisition times and enable going to well-defined one-photon excitation processes for triggering the structural dynamics of interest. This approach is paving the way to the development of a versatile tabletop x-ray source for femtosecond crystallography of small-unit-cell crystals.

The major advantage of x-rays over electrons in femtosecond time-resolved diffraction experiments is in the study of biological systems. In this regard, the spatial transverse coherence of the femtosecond electron sources has not yet achieved the magnitude needed to both provide atomic resolution and be capable of studying unit cells beyond 6 nm, which is at the border of protein crystallography. New developments in photocathode materials will likely solve this problem, but there are fundamental limits to electron source brightness that will limit the size of protein systems that can be studied.

The interest in time-resolved studies of biological systems was the driving force for the introduction of co-crystallized photolabile caged compounds for triggering biochemical processes (*65*), as well as the breakthrough in time-resolved Laue diffraction (*51*). To fully resolve the functionally relevant motions, the most important advance in x-ray sources has been the relatively recent introduction of the X-ray Free Electron Laser (XFEL) at hard x-ray wavelengths (*3*, *9*). The average beam current and output power is similar to third-generation synchrotrons; however, the design principle uses compressed high-energy (10 GeV range) electron pulses to produce extraordinary gain within the undulator so that the radiated x-rays can be reduced to the few-femtosecond domain. Most important, the oscillating electron bunch radiates in phase to produce a spatially coherent x-ray beam. The high degree of transverse spatial coherence is what distinguishes this source from all other x-ray sources. The decrease in pulse duration, energy bandwidth, and increased spatial coherence correspond to an overall gain of several orders of magnitude in source brightness that can be well defined in terms of x-ray peak brilliance (photons/pulse/mm^2/$mrad^2$/.1% bandwidth) (*9*, *10*). Furthermore, the temporal duration and the number of x-ray photons per pulse are in the perfect range to provide single-shot, few-femtosecond time resolution to atomic motions (*3*, *9*). However, femtosecond time-resolved crystallography with atomic resolution has not been achieved to date (*11*). This particular use of XFELs is still very much in the development stage, akin to the early use of synchrotrons for x-ray protein crystallography (*66*).

What are the challenges? First, the beamline involves kilometer-scale linear accelerators to get the electron bunch up to the giga–electron volt range to enter the undulator. The resultant x-ray pulses must be synchronized with the laser system used to trigger the structural dynamics within the required femtosecond time resolution or overall relative pathlength variations of less than 100 μm on this kilometer scale. There is a time stamping tool in which a reference response function to the x-ray pulses is used to define the time origin to retrieve ~50 fs time resolution, and higher resolution is possible. The other challenge is that the x-ray gain involves effectively a traveling wave amplifier rather than a resonator, as in an optical laser. The gain is far from being depleted as it would be in a normal laser oscillator, and the initial x-ray photon amplification cascade is initiated from noise in the radiated field. The source represents a self-amplification of spontaneous emission (SASE) source. There are very large (100%) fluctuations in x-ray pulse output and substantial modulation in x-ray spectrum. Seeding greatly reduces the amplitude noise (*3*) and will likely be in routine use in the near future. In addition, the spectrum is very narrow-band as compared with synchrotrons so that only a small fraction of reciprocal space is sampled, and many peaks for a given crystal orientation will be partials, meaning they are off the Bragg condition for completely constructive interference in the diffraction process (*3*).

These obstacles to femtosecond time-resolved crystallography have been overcome through shot-to-shot normalization, time stamping tools, high-throughput sampling, and new data analysis methods. The experiments are more demanding than conventional femtosecond laser spectroscopy. At present, these experiments require an expert user base to further develop the methodology. The most challenging problem may ultimately be the enormous number of crystal orientations and demands on sample for this class of experiment. For calibration, a typical protein crystal structure determination at a synchrotron facility requires on the order of 100 different orientations. For a narrow band source such as an XFEL, the number of required projections increases accordingly. Now consider that ideally, one would like 100 time points for sufficient dynamic range to the atomic motions. In the general case of irreversible sampling, each sampled spot is damaged by either the x-rays or by the femtosecond laser excitation to trigger the structure changes. Femtosecond time-resolved x-ray crystallography then requires a minimum basis of ~10,000 crystals or sufficiently large crystals to accommodate this large number of shots in order to provide adequately sampled reciprocal space and dynamic range to give atomic-level movies of the structural dynamics of interest. At the very least, the experiment requires two-orders-of-magnitude-higher sampling over conventional crystallography to give the time base.

The sample issues may seem to be an insurmountable problem. However, the use of large crystals and orthogonal beam geometries between the laser excitation pulse and x-ray probe pulse solves the problem of sufficient sample area and mismatch between the laser-excited volume and x-ray–probed volume (*53*). The time resolution is nominally sub-picosecond with this beam geometry because of transit time differences between the x-ray probe and laser excitation pulses. For highest time resolution, one would like to use near-collinear beam geometries. This geometry then requires crystal dimensions on the 10-micrometer scale or less for highest time resolution and to avoid excessive peak power conditions for the excitation. In this case, thousands of crystals are needed.

As mentioned above, one particularly ingenious solution for the use of XFELs in the study of nanocrystals involves aerosol and high-pressure liquid injectors (*3*). Nano- to micrometer-sized crystals are shot out of a nozzle under pressure

and hydrodynamic focusing in order to give a well-defined stream of crystals that can then be sampled by the x-ray beam downstream. This methodology has gone beyond proof-of-principle experiments with the high-resolution structure determination of the disease-causative agent in sleeping sickness that to date has only been successfully grown in vivo as microcrystals (*67*). As discussed in the accompanying article (*10*), the importance of the high brightness of the XFEL is that the diffraction can be attained before the onset of x-ray–induced damage so that the highest-quality diffraction is attained, limited only by the crystal quality. The use of this approach has also been recently demonstrated for time-resolved crystallography on the microsecond time scale. The timing in this case is determined by the travel time of the crystal along its flight path from the point of laser excitation to the point of the x-ray beam used for sampling the crystal structure (*3*, *68*). Normally, one needs to collect a reference diffraction pattern (no laser excitation) to index the crystal orientation and then compare with the diffraction pattern attained with laser excitation at some fixed time delay (laser on) in order to determine the changes in diffraction intensities after all the proper normalizations. This experiment relied solely on collecting enough data, in which the excitation was high enough to excite all the molecules within the crystal.

The first experiment focused on the important question of the transient structures of the photosystem 1(PS-1)/ferrodoxin system involved in the photoreduction of ferrodoxin as part of the solar energy transduction processes in plants. Structural changes were observed on the 5- to 10-microsecond time scale based on the change in amplitudes and baseline of what is an effective powder diffraction pattern [(*68*), figure 3D]. There were not enough diffraction patterns that could be indexed to invert to structure. Nevertheless, this result illustrates that long-time dynamics can be obtained this way, in which long laser excitation pulses can be used to excite 100% of the crystal. It is not clear that this approach will be suitable for femtosecond time resolution, in which only fractional excitation is generally needed in order to avoid excessive peak powers in the laser excitation. A reference diffraction pattern is important in this limit. An alternative approach that has been recently demonstrated is to use Si nanofabrication methods to make a crystallography chip that is capable of self-assembling thousands of crystals in seconds on an array of specifically designed features so as to spatially localize the crystal and introduce random orientations (*4*, *5*). This solution may also facilitate the use of synchrotron microfocus beam lines for high-throughput protein crystallography. Most important for time resolved studies, it enables a reference diffraction pattern to be collected. The basic principle of loading the crystallography chip was demonstrated by using fluorescently labeled polystyrene particles 2 μm in diameter [(4), figure 4]. For this size of particle, the features of the chip can be used for size exclusion, and the density of the chip can approach 1 M-crystal pixels/cm^2 with 75% fill factors. This concept uses the least amount of material possible, which is a critical consideration for precious protein crystals. There are a number of experiments in the pipeline that use different sample delivery systems, and one can expect groundbreaking experiments to be reported in the coming year.

The extension of femtosecond time-resolved crystallography to the study of protein dynamics will be an important development. In this regard, proteins have evolved to control barrier heights and thereby optimally control the transduction of stored chemical potential into functions. It is the passage over the barrier (on 100-fs time scales) that inextricably links chemistry to biology. In chemical terms, this problem becomes extremely interesting in terms of scaling chemistry to the next length scale of molecular synthesis. Taking into consideration the enormous reduction in dimensionality that occurs in relatively simple molecular systems, how can we make any sense of biological systems? The number of nuclear degrees of freedom for one of the simplest biological systems that serves as our benchmark for molecular cooperativity is the binding of oxygen to heme proteins (Fig. 5). This problem involves literally thousands of degrees of freedom. The chemistry aspects occur at the binding site. Here, one can define the

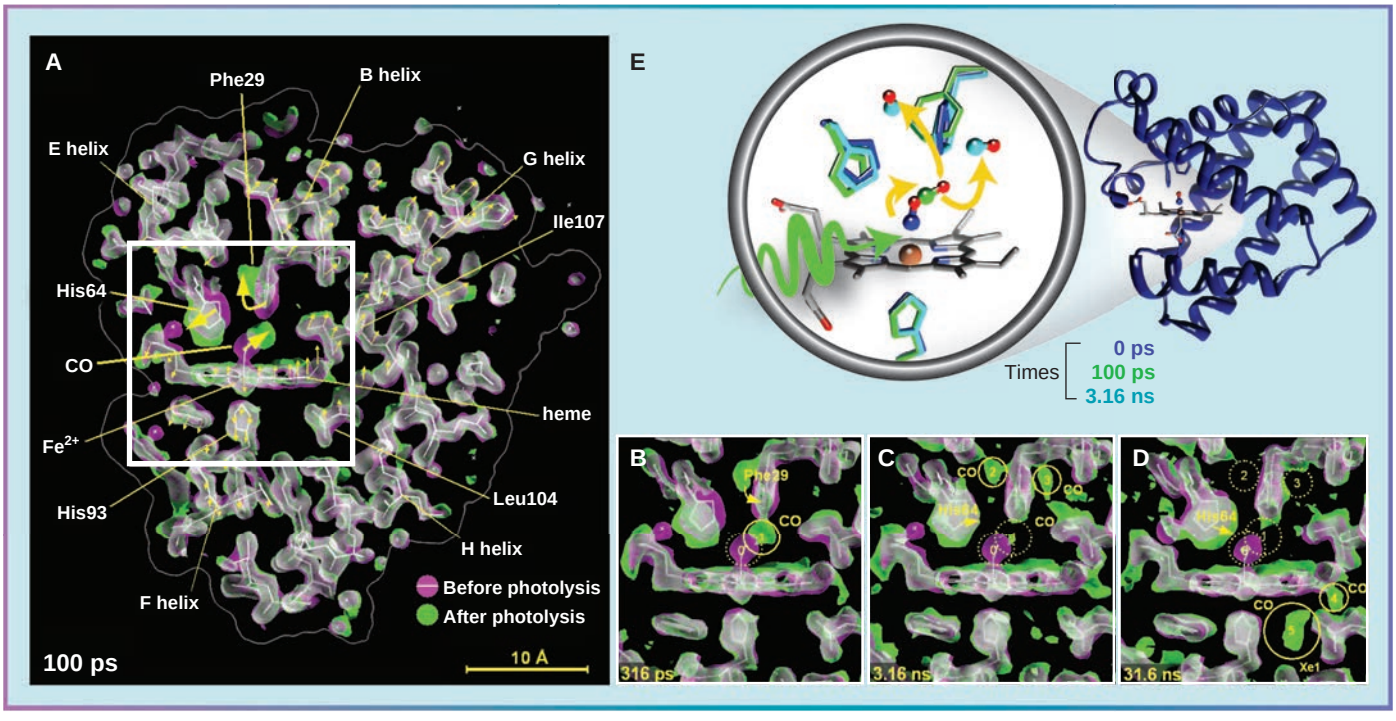

Fig. 5. Chemistry driving functionally relevant protein motions. (**A** to **D**) The structural changes following photodissociation of the CO ligand of carboxymyoglobin are shown at various time points, as indicated by the color gradients in the electron density maps [from (*53*)]. These motions are shown schematically in (**E**) using the corresponding protein data bank files 2G0V (100 ps) and 2G10 (3.16 ns). The earliest time point at 100 ps illustrates that the CO ligand has moved 2 Å from its binding site after bond breaking, along with substantial protein motions not resolved within this time resolution.

active site as the chemical system and the surrounding protein as the bath. The big questions surround the coupling between the chemistry at the active site and the surrounding protein that leads to changes in protein structure, which cascades into molecular feedback control of coupled chemical reactions and biological functions (*69*–*71*). On the basis of the observed time scales, biological systems clearly course grain sample their potential energy surface to direct chemical energy into functions (*72*). Nature has highly optimized the system-bath coupling to take advantage of the inherent correlations imposed on the evolved structures. In this regard, the importance of collective modes in describing stochastic fluctuations of proteins have long been identified (*73*–*76*). There is also experimental evidence that has led to the collective mode-coupling model to explain the relationship between the chemistry and the functionally relevant motions encoded in protein structure (*69*, *70*, *77*). These anharmonic motions are highly damped to overdamped relaxation modes and are not amenable to spectroscopic investigation. The direct observation of the highly correlated motions involved in biological response functions will give us our most fundamental (atomic-) level basis to understand the structure-function correlation in biological systems.

How close are we to this goal? Shown in Fig. 5 is the high degree of information available with full atomic resolution in catching protein structural changes. This particular study (*53*) is representative of a number of related studies (*50*, *51*, *78*). The time resolution in following the dynamics of CO dissociation in myglobin in this case was 150 ps (*53*). One can clearly see highly localized changes involved in the ligand dissociation and spatial transport out of the protein. The motions appear to be localized, but there is a high degree of steric coupling between the protein fluctuations and motion of even a simple diatomic molecule. The structural changes must involve correlated motions over some unknown length and time scales. Imagine if we could watch the chemistry unfold at the active site and its coupling to the protein motions on the 100-fs time scale to catch these details. We are getting close.

Summary and Future Outlook

The technical challenges posed for the development of ultrabright electron and x-ray sources for the observation of atomic motions have been met. As we enter this new age of atom gazing, we have already been able to see the enormous reduction in dimensionality in barrier-crossing regions that makes chemical concepts transferrable from one molecule to another. We now have the tools to directly observe the far-from-equilibrium motions that lead to chemistry. Each class of chemical reaction will have a distinct power spectrum related to the key modes that most strongly couple to the reaction coordinate. It is early days, but it may be possible to one day categorize these far-from-equilibrium reaction modes in much the same way that we discuss normal modes in vibrational spectroscopy in relation to equilibrium fluctuations. These new advances will provide the benchmarks for driving progress in time-dependent ab initio theoretical methods in order to understand chemistry on a grander level. Additionally, these new insights will equally improve our understanding of the structure-function relationship in biological systems and ultimately may lead to a systematic basis for categorizing protein structural motifs in terms of controlling the system bath coupling—coupling between active sites and the surrounding protein. At this point, we will be able to connect structure to dynamics in improving rational control of chemistry from synthesis to drug design.

The primary events of chemistry can now be studied at the atomic level of inspection over the relevant length and time scales, and an atomic-level basis for understanding the connection between chemistry and biology is in sight. Improvements in source brightness will continue as needed. The real challenge is the development of systems that can be optically triggered to probe different aspects of chemistry and biology.

Crystallography brought us our first atomic pictures of matter. With brighter sources, it is now enabling the connection between structure and dynamics in how things work at the atomic level of detail. Not being able to resist the pun, the future of femtosecond crystallography is bright indeed.

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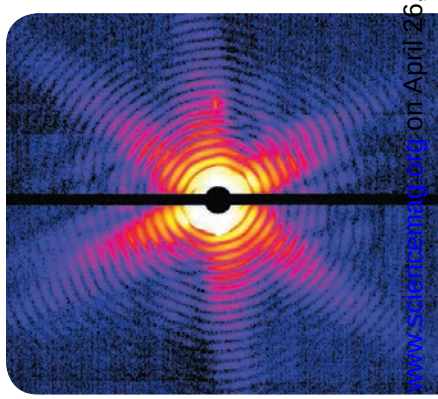
Gautam R. Desiraju is a professor of chemistry at the Indian Institute of Science, Bangalore, India, and president of the International Union of Crystallography. E-mail: desiraju@sscu.iisc.ernet.in

Crystallography and Geopolitics

DEVELOPED AND DEVELOPING NATIONS RECOGNIZE THAT INNOVATION IS KEY TO THEIR ECONOMIES. Connecting this with the discipline of crystallography may not seem immediately apparent, but during the past century, understanding the structure of matter has transformed industries and created new frontiers, from the design of new medicines and materials to assessing the mineral content of Mars. The future global economy will be determined by progress in cutting-edge fields. However, the playing field is not level in crystallography, which is why the International Union of Crystallography (IUCr) and the United Nations Educational, Scientific and Cultural Organization (UNESCO) have marked 2014 as the International Year of Crystallography. The aim is to improve public awareness of the field, boost access to instrumentation and high-level research, nurture "home-grown" crystallographers in developing nations, and increase international collaborations for the benefit of future generations.

The development of scientifically influential ideas is most prominent in wealthy countries. Those nations should continue to invest in science to remain economically advanced. They should not try to live off their existing scientific capital and hope to compensate for future shortfalls through business, management, and outsourcing to ostensibly "cheaper" countries. A developing country, on the other hand, needs to invest in science to define its own technologies and find a voice in international forums. But any country, wealthy or not, that lacks a healthy native scientific enterprise cannot make up the deficit by importing science from more scientifically advanced nations. Such attempts can never lead to a stable scientific culture or society. Embracing the relevance of science in one's life and growing science locally are the true measure of a country's scientific success, not the number of Nobel Prizes that have been given to people who were born, lived, or worked in that country.



The newly advancing economies of Brazil, Russia, India, China, and South Africa (the so-called BRICS nations) are investing heavily in science and technology. As a result, crystallography's future may well lie in these parts of the world, which have people power and increasing economic muscle. By 2030, China, India, and the African continent will have 1.5 billion people each, most of whom will be educated. All of the Western world will by then have just 1 billion people. This means that "Chindiafrica," with its 4.5 billion people, could exert a substantial geopolitical and scientific influence in the world, with the focal point being the Indian Ocean rather than the northern Atlantic.

The International Year of Crystallography has placed a special focus on Africa, Latin America, and Asia. The efforts include a plan for "open laboratories" that, in partnership with industry, will enable students in far-flung lands to have hands-on training in modern techniques and expose them to cutting-edge research in the field. Open labs in Uruguay, Ivory Coast, and Algeria are already on the anvil. The IUCr also is running a training program in crystallography, in which students from sub-Saharan Africa can obtain a Ph.D. in the field in more advanced locales, such as the Universities of the Witwatersrand and Cape Town in South Africa.

More-powerful synchrotrons and free-electron laser facilities will be needed to determine increasingly complex structures. IUCr and UNESCO hope that setting up such facilities will assist in expanding and strengthening crystallography beyond 2014. A good example of this is in Jordan, where governments are working together to construct the Synchrotron-light for Experimental Science and Applications in the Middle East (SESAME). Brazil has impressive synchrotron facilities where collaboration among scientists from other Latin American countries is encouraged. More forums to guide research priorities, multinational partnerships, and funding arrangements are needed. What is most important is for scientists to interact seamlessly with the enormous amounts of data that will be generated in crystallography so that anyone, anywhere, can get any kind of structural information and use it profitably. Crystallography is a facilitating discipline, and this is why it will always endure.

– Gautam R. Desiraju

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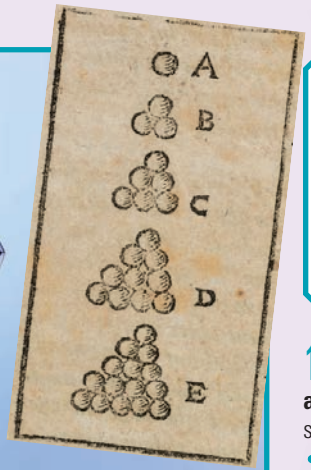
Dazzling History

Over the past century, x-ray crystallography has transformed scientists' understanding of the structure and behavior of materials

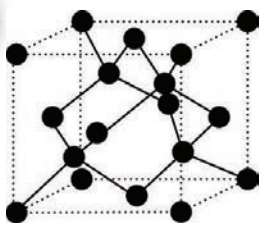
REPORTED AND WRITTEN BY THOMAS SUMNER

PREHISTORY



1611

Johannes Kepler speculates that **snowflakes** are **hexagonal grids** of water particles—a hypothesis that cannot be tested for centuries to come.



1913

Braggs determine crystal structure of **diamond**.

1895 1901 Physics

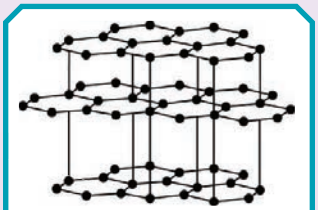
Wilhelm Röntgen produces and measures **x-rays**.

1912 1914 Physics

Max von Laue creates a **diffraction pattern** by firing x-rays at a crystal of copper sulfate but cannot interpret it.

1916

Powder diffraction analysis makes it possible to study small crystals.

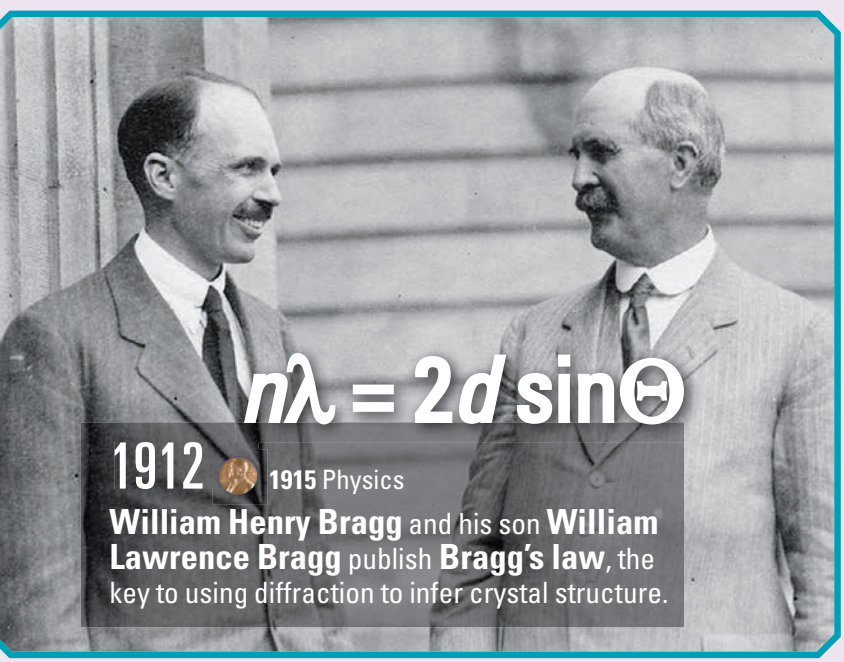


1924

John Desmond Bernal determines structure of **graphite**.

1937 1946 Chemistry

James Sumner demonstrates that any protein can be **crystallized**.



$$n\lambda = 2d\sin\Theta$$

1912 1915 Physics

William Henry Bragg and his son **William Lawrence Bragg** publish **Bragg's law**, the key to using diffraction to infer crystal structure.

1945 1964 Chemistry

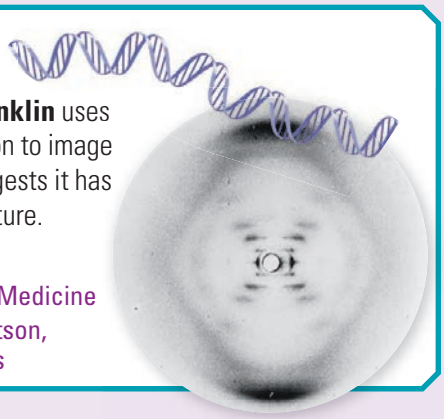
Dorothy Hodgkin and colleagues determine structure of **penicillin**, the first complex molecule solved by x-rays.

1946 1994 Physics

First **neutron diffraction** experiments; the technique provides **3D structures** and other details that x-rays cannot.

1952

Rosalind Franklin uses x-ray diffraction to image **DNA** and suggests it has a helical structure.



1962 Physiology or Medicine
F. Crick, J. Watson, and M. Wilkins

1952

Grazing-incidence optics paves way for modern x-ray studies.

1958 1962 Chemistry

John Kendrew and Max Perutz determine first protein structures, of **myoglobin and hemoglobin**.

1970

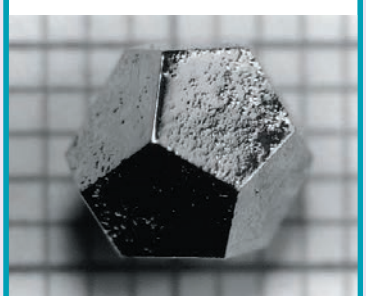
The first **synchrotron x-ray** sources open, producing brilliant x-rays for detailed crystallography research.

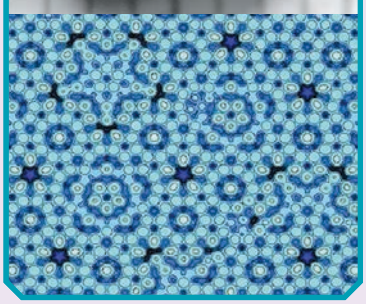
1978

Tomato bushy stunt virus is imaged—the first viral structure mapped at atomic level.

1982 2011 Chemistry

Scientists observe first **quasicrystals**, strange materials whose atoms follow an **ordered but nonrepeating pattern**.





1984 1988 Chemistry

Researchers solve structure of **photosynthesis reaction site**.

1989

Time-resolved crystallography reveals action mechanisms of rapidly changing molecules.

1990s

Automated protein crystallization. Number of structures in the Protein Data Bank grows from 507 in 1990 to 97,980 in 2014.

2000

Protein Structure Initiative begins (see News Focus, in this issue).

2001

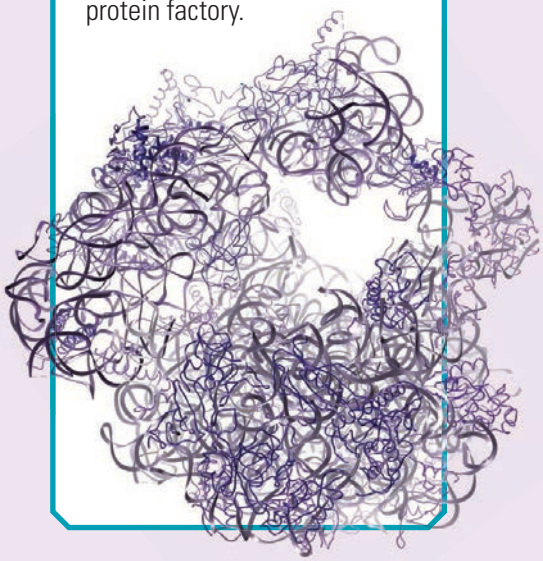
"Robotic beamlines" start to speed sample analysis at x-ray sources.

2002

Microfluidic chips promise to boost automated protein-crystal growing.

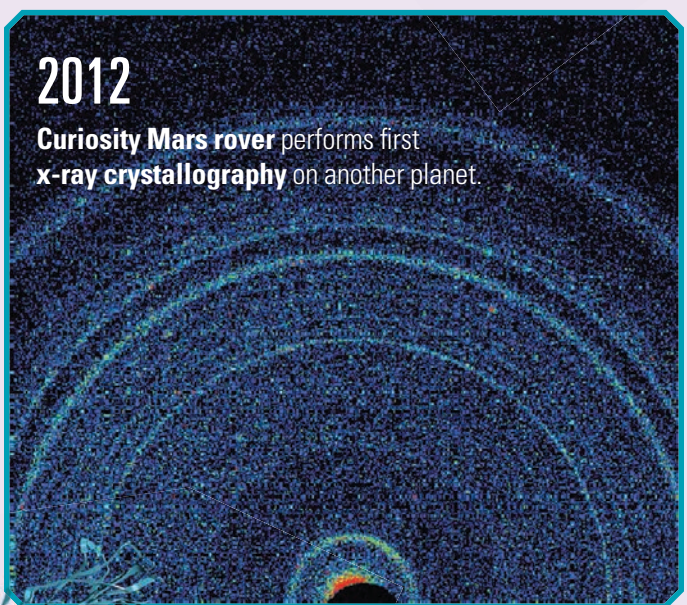
2000 2009 Chemistry

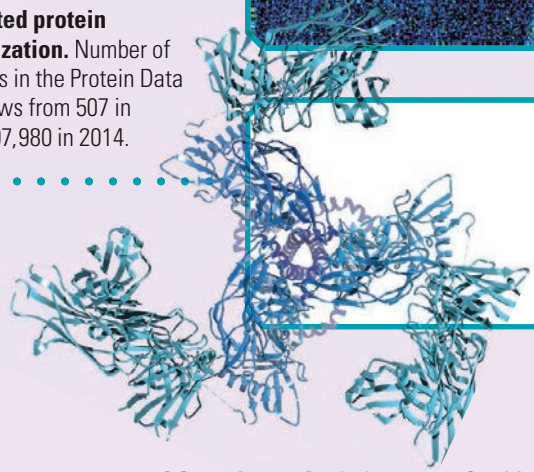
Scientists solve structure of a **ribosome**, cells' protein factory.



2012

Curiosity Mars rover performs first **x-ray crystallography** on another planet.





2013

Crystallography yields a detailed picture of the protein that **HIV** uses to **invade immune cells**.

Nobel Prize awarded for work

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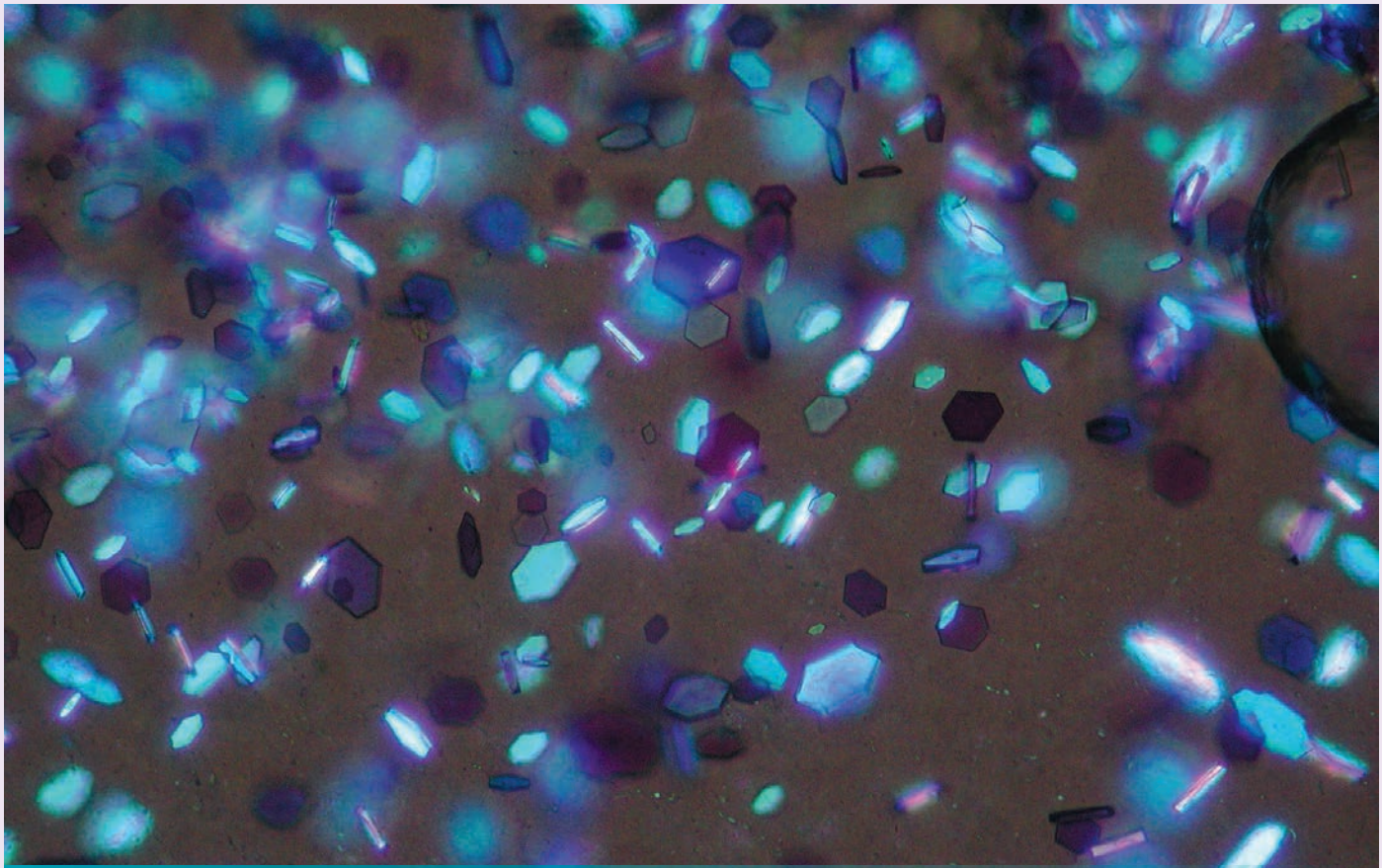


Gently Does It

A technique for crystallizing fragile biomolecules without disrupting them is helping researchers probe the structures of some of the body's most important but elusive proteins: those that usher other chemicals through the cell membrane

The tiny purple crystals, glistening within a translucent, fatty gel, signaled that Ehud Landau and Jürg Rosenbusch had made headway on one of the toughest problems in x-ray crystallography. To map a protein's atomic structure using x-rays, crystallographers have to coax its molecules to align themselves in crystals, like soldiers in perfect formation. That's difficult enough for ordinary proteins, which are complex, flexible molecules. But the membrane proteins that straddle the cell's surface and control the chemical traffic in and out are an even bigger challenge. Nestled within their normal protective environment, membrane proteins are stable and well-behaved. But take them out to try and get them to line up, and the task is like herding cats.

Two decades ago, Landau, a chemist then at the University of Basel in Switzerland, thought the answer might lie in a curious mixture of fatlike molecules called lipids, blended with water and other compounds. The concoctions spontaneously form 3D shapes called the lipidic cubic phase (LCP), and Landau hoped they could serve as a synthetic cell membrane to keep the membrane proteins happy outside cells. He and Rosenbusch, a structural biologist also at Basel, tested the scheme with a purple membrane protein known as bacteriorhodopsin (bR), found in halobacteria. The plan worked. The result was the 50-micron-wide bR crystals—and, in the years that followed, a mini-explosion in membrane protein crystal structures.

Membrane proteins may be the most important molecules in biology. These enzymes, receptors, channels, and transporters account for more than half of the targets for all pharmaceutical compounds on the market. And LCP has been essential for understanding them. "It's been magical

CREDIT: VADIM CHEREZOV/THE SCRIPPS RESEARCH INSTITUTE

Crystal power. Crystallizing proteins such as bacteriorhodopsin is key to solving their atomic structure.

for us," says Wayne Hendrickson, a protein crystallographer at Columbia University, who has recently used the technique to solve two membrane protein structures.

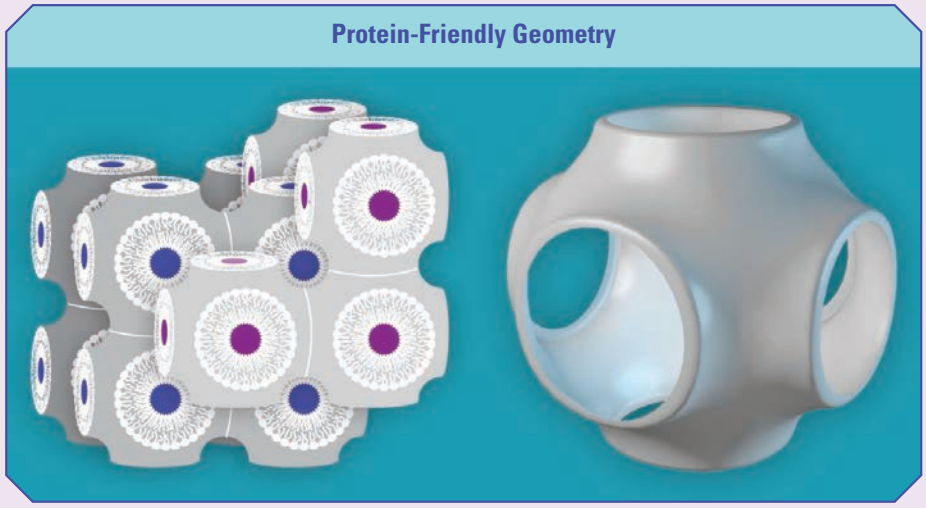
But getting the LCP mixtures right and handling them is tricky. After their first glimpse of those purple bR crystals, it took Landau and Rosenbusch several more years of tinkering before they could nail down the first high-resolution structure of the protein (*Science*, 12 September 1997, p. 1676). Now, however, thanks to decades of painstaking work by a small band of researchers, the technique is beginning to hit its stride.

Fits and starts

LCP wasn't the first technique that crystal growers used to enforce order among membrane proteins. Nor is it the most common approach even today. Both of those honors go to a technique that uses soaplike detergents to purify membrane proteins and get them out of cell membranes, a necessary step for getting them to crystallize.

Detergents contain two different kinds of compounds joined at the hip. On one end are hydrophilic groups, which readily associate with water. On the other end are fatty hydrocarbon chains. Dump the detergents into water in the right conditions and they form micelles, tiny spheres with the hydrophilic portion facing out into the water and the fatty hydrocarbon tails pointing inward to minimize their interaction with water. When lipid molecules, which have different hydrophilic groups linked to hydrocarbon tails, are added, the mix can form "bicelles" shaped like tiny disks made from a combination of the lipids and detergents, all with their hydrophilic portions facing out into the water.

Membrane proteins also typically contain one portion that prefers to associate with fatty membrane molecules, and two others that gravitate to the watery environment outside or inside the cell. So if you toss a membrane protein into a solution with micelles or bicelles, the water-fleeing portions of the protein will wedge themselves into the friendly confines of the hydrocarbons, stabilizing their structure. Add millions of copies of the same membrane protein, and if you're lucky they will all orient themselves the exact same way, making it possible for them to pack into an orderly crystal.



Shapely. In a lipidic cubic phase structure, lipid molecules form a hollow framework (*right*) that extends to form a 3D grid around water channels (*left*, purple and blue).

That strategy works in some cases. But often it goes spectacularly wrong. Sometimes the detergents are too harsh and rip apart the proteins. The tightly curved spherical micelles can wrench the proteins out of their normal shape, and subtle temperature differences can wreak havoc with bicelles.

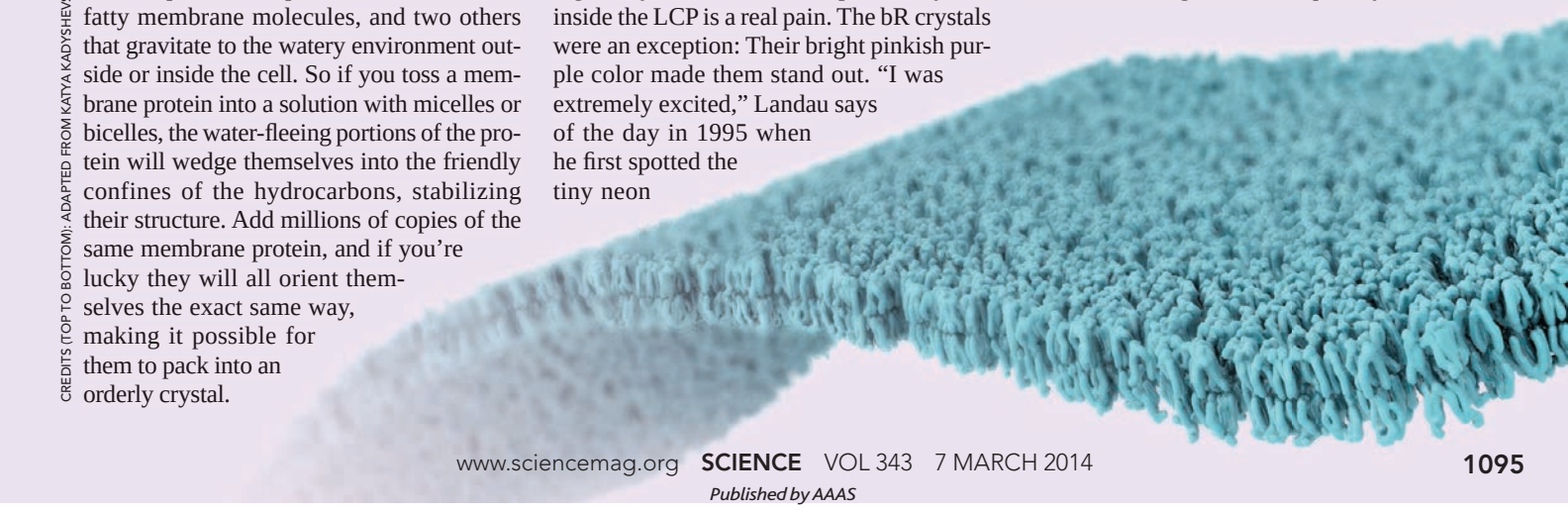
Back in 1992, Landau thought LCPs might be a gentler option. LCPs have a gradually curving framework that arranges itself into a 3D grid surrounding a network of watery channels (see figure, above). Landau and Rosenbusch hoped the LCPs' combination of the lipid framework and watery channels would keep both parts of membrane proteins happy and the 3D grid arrangement might help orient them all in the same direction.

But LCPs "can be a hassle to work with," says Martin Caffrey, an LCP expert and membrane protein crystallographer at Trinity College Dublin. LCP is a clear goop with the consistency of toothpaste, Caffrey explains. While crystals can simply be filtered out of liquid detergent solutions, finding nearly invisible flecks of protein crystals inside the LCP is a real pain. The bR crystals were an exception: Their bright pinkish purple color made them stand out. "I was extremely excited," Landau says of the day in 1995 when he first spotted the tiny neon crystallites. "It was obvious to me that our concept had worked."

Of course, Landau and his colleagues still didn't have a structure. And their next problem was the x-ray beams produced by synchrotrons. These stadium-sized machines fire a staccato burst of densely packed x-rays at their targets. By tracking the way the x-rays diffract off their target, researchers can deduce the atomic structure of the material.

The trouble was that the LCP-grown bR crystals were significantly smaller than those produced in detergent micelles. Most synchrotron beams at the time were 100 microns across, or more—twice the width of the bR crystals. That meant that most of the x-rays in the beamline would whiz right by the bR crystallite and contribute nothing to the diffraction pattern.

Then fortune smiled: The newly built ESRF synchrotron in Grenoble, France, had just opened its first microfocus beamline for work on just such tiny crystals. Landau and Rosenbusch applied for time on the beam, got it, and quickly nailed



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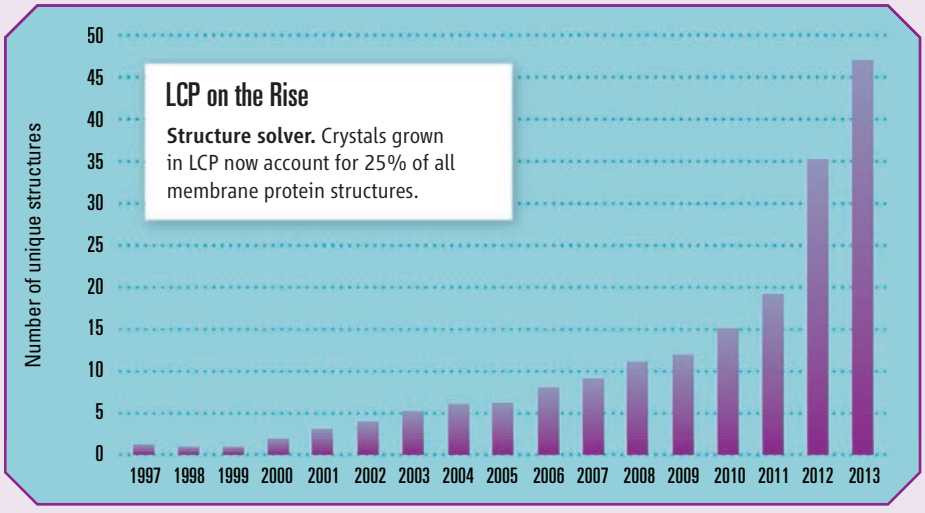
down a crisp diffraction pattern for the protein. "This was an extraordinary breakthrough," Caffrey says.

The question was whether the approach would work for other membrane proteins. Through the late 1990s, Rosenbusch, Landau, and others produced a string of successful x-ray structures with other colored membrane proteins, such as the greenish photosynthetic reaction center. "After that it got quiet," Caffrey says. Growth conditions that produce protein crystals in LCP invariably trap myriad tiny bubbles in the gel as well, making it even harder to pick out the crystals, if they were there at all.

Caffrey, then at Ohio State University, Columbus, set out to speed things up. In 2000, he and Vadim Cherezov, a postdoc, set about inventing new tools to speed the discovery of crystals in LCP. One was a "sandwich plate" that squished blobs of the clear goop between two glass plates, to make crystals easier to spot under a microscope. Another was a robot that automated the mixing of different lipids, salts, and buffers needed to crystallize each protein.

Despite a couple of years of rapid progress, LCP efforts nearly ground to a halt again in 2003 when Caffrey was recruited away from Ohio State to form a group dedicated to LCP and membrane protein crystallography at the University of Limerick, in his native Ireland. U.S. science funding agency rules stated that Caffrey was unable to take his robot and other equipment that had been paid for by U.S. taxpayers. Cherezov faced an uncertain future as well. But things took a welcome turn when Cherezov went to San Diego, California, to visit a friend who worked at the Scripps Research Institute. There he met Raymond Stevens, a renowned structural biologist. After inviting Cherezov to give a seminar on LCP, Stevens asked him to join his group.



"Something that no one has ever seen"

The new landing spot was an ideal fit. At the time, Stevens was collaborating with Brian Kobilka, a biochemist at Stanford University, on attempts to crystallize membrane proteins known as G protein–coupled receptors (GPCRs). GPCRs are one of medicine's most important sets of membrane proteins, as they transfer chemical signals from outside cells to G proteins inside cells. The G proteins, in turn, launch a variety of molecular dominoes that govern everything from your heart rate to your sense of smell.

By the mid-2000s, Kobilka had managed to grow crystals of a GPCR known as the β2 adrenergic receptor (β2-AR)—a cell-signaling component involved in everything from heart muscle contraction to digestion—in conventional lipid micelles. But the crystals were poor and didn't diffract well, Kobilka says. Like many other membrane proteins, β2-AR is a Janus molecule. The part that prefers to nestle within the fatty membrane usually keeps an orderly and stable structure. But the section that protrudes into the watery surroundings flops around like a flag in the wind. Kobilka's lab was struggling to find ways to stabilize those floppy portions to ensure that all copies of the protein lined up in the same manner inside a crystal. The researchers got partway there by adding copies of an antibody that grabbed part of the floppy portion of the β2-AR and held it in place. Then they grew the protein-antibody complexes in bicelles. The result, published in *Nature*, was one of the first crystal structures of a GPCR. The crystal difracted to 3.4 angstroms, a resolution that reveals most of the protein's amino acids.

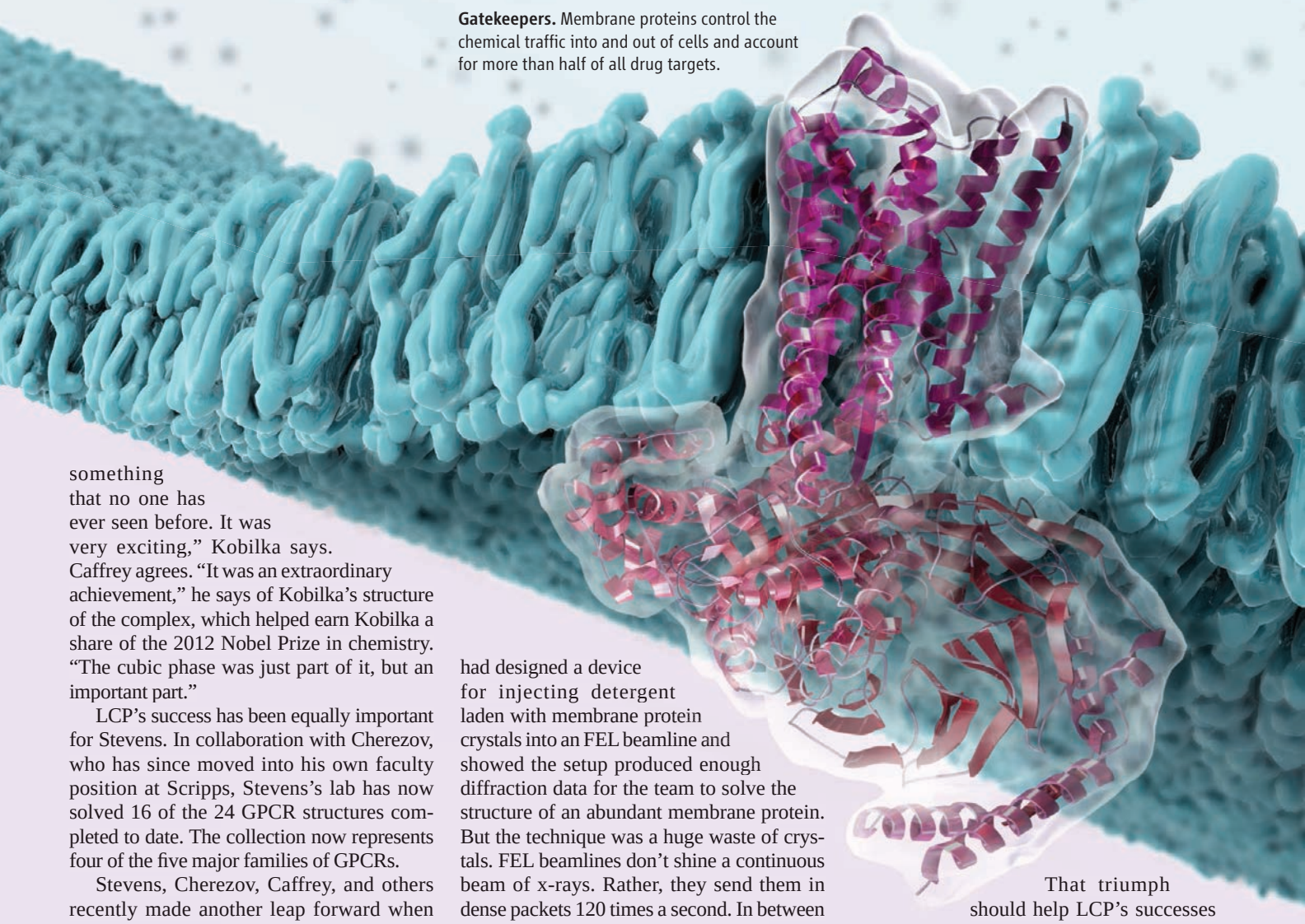
In hopes of seeing even more detail, Kobilka and colleagues tried another tack. They clipped off a particularly unwieldy portion of β2-AR and replaced it with an orderly protein called T4 lysozyme, and grew those hybrids in bicelles. This got them crystals that diffracted to 4.2 angstroms. So they sent a batch of these hybrid membrane proteins to Stevens's lab. After a few months spent optimizing the LCP conditions, Cherezov produced high-quality crystals, and the researchers took them to the microfocus beamline at the Advanced Photon Source at Argonne National Laboratory in Illinois. The result was a 2.4 angstrom resolution structure (*Science*, 23 November 2007, p. 1258), which *Science* named one of its top 10 breakthroughs of the year.

Next, Kobilka wanted to see if he could get the structure of a GPCR bound to its G protein mate, which would show the GPCR's conformation in its "on" state. But Stevens, and his postdoc Cherezov, wanted to explore the broader landscape of GPCRs; humans alone have an estimated 800 varieties. So Kobilka teamed up with Cherezov's former mentor, Caffrey. The G protein turned out to be a behemoth, roughly twice as big as the GPCR. That made it too big to fit into the 50-angstrom-wide watery channels in the LCP. Kobilka hoped to find a way to make the channels bigger.

Back when Caffrey was at Ohio State, he had experimented with dozens of different lipids, charting their effect on the shape and size of the LCP network. He told Kobilka he thought they could widen the channels by replacing the conventional lipid in LCP, known as monoolein, with a shorter chain lipid known as 7.7 MAG.

Caffrey was right. In 2011, using 7.7 MAG for their LCP, along with other changes, Caffrey, Kobilka, and their colleagues were able to get crystals of the complex and work out the structure. "There have been three to four times in my career where I have seen

CREDITS (TOP TO BOTTOM): (ILLUSTRATION) V. ALTOUNIAN/*SCIENCE* BASED ON PROTEIN STRUCTURE FROM BRIAN KOBILKA; (DATA SOURCE) VADIM CHEREZOV/THE SCRIPPS RESEARCH INSTITUTE



Gatekeepers. Membrane proteins control the chemical traffic into and out of cells and account for more than half of all drug targets.

something that no one has ever seen before. It was very exciting," Kobilka says. Caffrey agrees. "It was an extraordinary achievement," he says of Kobilka's structure of the complex, which helped earn Kobilka a share of the 2012 Nobel Prize in chemistry. "The cubic phase was just part of it, but an important part."

LCP's success has been equally important for Stevens. In collaboration with Cherezov, who has since moved into his own faculty position at Scripps, Stevens's lab has now solved 16 of the 24 GPCR structures completed to date. The collection now represents four of the five major families of GPCRs.

Stevens, Cherezov, Caffrey, and others recently made another leap forward when they adapted a beamline at the free-electron laser (FEL) at the Center for Free-Electron Laser Science in Hamburg, Germany, to solve structures of LCP-derived crystals of membrane proteins with unprecedented efficiency (*Science*, 20 December 2013, p. 1521). FELs represent the latest in synchrotron technology, able to produce x-ray beams that are tighter and pack more than 1 billion times more photons into a given area than ever before. The beams are so powerful, in fact, that they vaporize crystals as soon as they hit them. But because the x-ray photons are traveling at the speed of light, they still manage to diffract well before the slow-moving atoms in the crystal explode outward.

The trick is zapping enough crystals to build up sufficient data to solve a protein's structure. In 2011, researchers led by Henry Chapman at the Center for Free-Electron Laser Science and Petra Fromme and Uwe Weierstall at Arizona State University, Tempe, had designed a device for injecting detergent laden with membrane protein crystals into an FEL beamline and showed the setup produced enough diffraction data for the team to solve the structure of an abundant membrane protein. But the technique was a huge waste of crystals. FEL beamlines don't shine a continuous beam of x-rays. Rather, they send them in dense packets 120 times a second. In between those bursts is essentially dead space that produces no data. To ensure that the x-ray bursts would hit enough crystals, Chapman's team had to spray in a steady stream of the detergent-and-crystal mixture. The x-ray packets hit only about one crystal in 10,000; the others produced no data. "It's hugely wasteful" and thus can't be used with most membrane proteins, which can be harvested only in tiny amounts, Caffrey says.

The LCP aficionados asked Fromme and her injection-builder colleagues to remake their injector to work with the LCP gel. A redesign worked. When the thick LCP goop is pushed through a tiny injector nozzle, it forms a continuous "stream" at a much lower velocity than the previous liquid stream, much as toothpaste emerges from a tube more slowly than a jet of water from a hose. The result was that far more crystals were hit by x-ray packets and the crystal losses were reduced between 100- and 1000-fold.

That triumph should help LCP's successes continue to roll in. Cherezov notes that in the past 2 years, structural biologists have solved more than 25 unique membrane protein structures with LCP—more than in all previous years combined. LCP-aided structures now account for 25% of all solved membrane structures, a fraction that is growing rapidly.

That doesn't mean the membrane protein crystallography challenge has been solved. "LCP is not a panacea," as it still doesn't work with some of the larger protein complexes, Cherezov cautions. But clearly, Stevens says, the logjam has broken. "For single membrane proteins, for the most part, if we want to get a structure we can get it," he says. With drugmakers now turning to membrane protein structures to identify novel targets for new classes of drugs against everything from pain and depression to heart disease and migraine headaches, LCP's success may soon make a difference in millions of peoples' lives.

–ROBERT F. SERVICE

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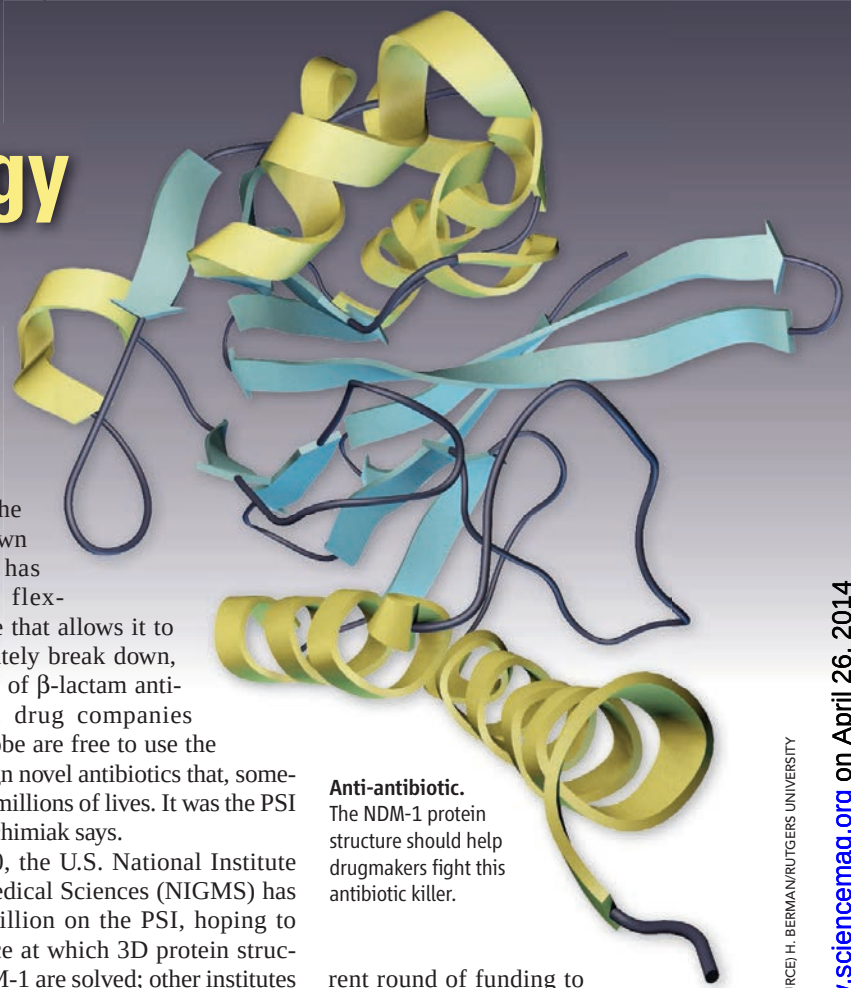
Structural Biology Scales Down

The United States is winding down a $1 billion project to churn out protein structures. What will that mean for the field?



Anti-antibiotic. The NDM-1 protein structure should help drugmakers fight this antibiotic killer.

CREDITS (TOP TO BOTTOM): V. ALTOUNIAN/*SCIENCE*; (DATA SOURCE) H. BERMAN/RUTGERS UNIVERSITY

THREE YEARS AGO, ANDRZEJ JOACHIMIAK decided to take on the superbugs. Infections from these antibiotic-resistant microbes are on an alarming rise globally, accounting for 2 million cases and 23,000 deaths a year in the United States alone. Among the most dangerous bugs are new strains with a protein known as NDM-1 that chops up a wide variety of previously effective antibiotics known as β-lactams, drugs that include penicillin.

Thanks to a long-running effort called the Protein Structure Initiative (PSI), Joachimiak had the tools to work out NDM-1's structure and pinpoint its weaknesses. Joachimiak, a structural biologist at Argonne National Laboratory in Illinois, and his colleagues used robots to synthesize 98 NDM-1 genes, each with subtle sequence variations. They succeeded in engineering bacteria to express 59 of those genes and produce their corresponding proteins at a high concentration. The researchers purified 53 of the proteins and coaxed 21 into forming crystals, many in combination with different druglike inhibitors and potential antibiotics. Then they shipped the best samples to the Advanced Photon Source, a stadium-sized synchrotron that fires a powerful beam of x-rays, bouncing them off crystalline solids to map their 3D atomic structures.

Joachimiak and his colleagues worked out 11 such atomic maps; others are still in progress. So far, the maps have shown that NDM-1 has an enlarged, flexible active site that allows it to fit, and ultimately break down, a wide variety of β-lactam antibiotics. Now, drug companies around the globe are free to use the results to design novel antibiotics that, someday, may save millions of lives. It was the PSI at its best, Joachimiak says.

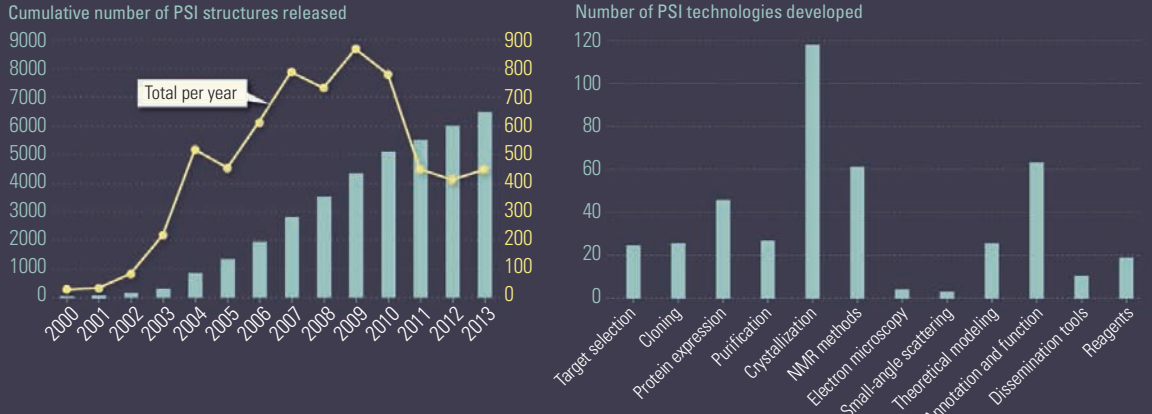
Since 2000, the U.S. National Institute of General Medical Sciences (NIGMS) has spent $907 million on the PSI, hoping to rev up the pace at which 3D protein structures like NDM-1 are solved; other institutes of the National Institutes of Health (NIH) chipped in another $23 million. That money funded large teams of biologists, physicists, chemists, and engineers to collaborate on not only determining protein structures, but also reinventing the way that this science is done. So far, PSI investigators have worked out the structures for 6507 proteins, 6.6% of all the structures with 3D data deposited in the international repository known as the Protein Data Bank (PDB).

But last fall, an NIGMS advisory council bowed to long-standing criticism of the PSI and pulled the plug on it, allowing its current round of funding to expire in June 2015. "In the current budget environment, in order to start a new program or bolster support for existing priorities such as investigator-initiated research, other programs must be adjusted or ended," NIGMS's new director, Jon Lorsch, wrote in a blog post in September 2013.

The announcement left longtime supporters of the PSI reeling and critics gleeful. But most of all, it has raised a string of questions: What was learned from the near $1 billion big-science experiment? What will happen to this team-oriented approach to biology? What will become of the high-speed facili-

PSI's History In Numbers

Ups and downs. The PSI cranked out protein structures and technologies. But rising costs squeezed competitive research grants.



ties that were created? And what does the PSI's demise mean for the future of structural biology in the United States? "Structural biology really is at a crossroads," says Raymond Stevens, a structural biologist at the Scripps Research Institute in San Diego, California, and the leader of a PSI center devoted to solving structures of cell membrane proteins. "The PSI is dead. I view it as an opportunity to think about what's next."

The hunt is on

Before the PSI, structural biology was painfully slow. Typically, individual labs worked for months or years to clone a gene for a particular protein into bacteria or yeast cells and purify it. Then they often tried adding countless combinations of salts, buffers, and other additives to their protein-laced solutions to coax the proteins to arrange themselves into tiny crystals. The good ones could then be blasted with x-rays to see whether they would diffract in a tight pattern. After that, researchers often spent additional months or years mapping out the atoms. By the late 1990s, the PDB contained structures of only about 10,000 proteins. Meanwhile, the Human Genome Project was about to inundate researchers with genes for all the million-plus proteins in the human body. Determining their 3D structures would be a key step in sorting out their functions—and biologists realized that they would have to pick up the pace or fall hopelessly far behind.

Enter the PSI. In 2000, NIGMS officials laid out the program's goals. First, develop the technology needed to solve 5000 structures in 10 years. Then learn how to bypass crystallography altogether by using the solved structures to develop computer models that could take the gene sequence of an unknown protein and compute its likely 3D shape, giving insights into its function.

From September 2000 through June 2005, NIGMS spent $265 million on a pilot program, automating each phase of protein structure determination, including expressing proteins, purifying them, crystallizing them, collecting diffraction data at synchrotrons, and using software to solve their structures. More than 1100 structures later, NIGMS officials decided that the effort had succeeded well enough to push for a second "production" phase, PSI-2. From July 2005 through June 2010, NIGMS spent $346 million on four large-scale high-throughput centers, six specialized centers focused on developing methods for solving more challenging structures, and a pair of computer modeling centers. All told, the effort generated another 3700 structures. Most were unique, meaning that they shared less than 30% of their genetic sequence with any other protein and folded in ways no other protein did.

But the PSI also churned out controversy. The bulk of the newly discovered proteins came from bacteria, and researchers knew little about their function. PSI researchers argued that the bacterial proteins were teaching them basic rules of protein folding. But biologists outside the PSI wondered why so much effort was being spent pursuing proteins unlikely to improve human health. In 2007, a midterm review of the PSI-2's progress, led by University of Michigan, Ann Arbor, structural biologist Janet Smith, concluded that "the large PSI structure-determination centers are not cost-effective in terms of benefit to biomedical research." The reviewers recommended that the PSI be revamped to target proteins of high interest to biologists.

NIGMS obliged and funded a third phase of the program, dubbed PSI:Biology. Gone was the talk of seeking out unique ways in which proteins fold and obtaining structures of representatives of each protein "family." Instead, the four high-throughput centers and an additional nine centers refocused their efforts on solving biologically important structures.

Still, criticisms persisted. In a midterm evaluation of the PSI's third phase produced last year, yet another outside panel of biologists faulted the high-throughput centers. "[M]any of the projects being developed are technology driven, chosen because they can capitalize on the existing high-throughput structure pipelines, rather than being driven by biological interest or impact," the report stated. The panel recommended continuing PSI:Biology for another 3- or 5-year term beyond 2015. But it also advised NIGMS to begin thinking about how best to end the program and move structural biology away from a dedicated source of set-aside funding.

Crystallography at 100

See also the special section on crystallography starting on page 1091.

Disputed legacy

Lorsch and an NIGMS advisory panel jumped at the recommendation. They decided to forgo another phase and prepare right away for the transition, creating panels to work out what to do with the current PSI centers and all the equipment and technologies they have produced, and how best to fund structural biology going forward.

Opinions about NIGMS's decision are mixed. "The PSI was a bad idea from the start," says Stephen Harrison, a structural biologist at Harvard University and a longtime critic of the PSI. The initiative did speed technology development, he says, but much of that progress probably would have taken place anyway. Now that the program is being terminated, Harrison says, "structural biology can now go on where it should have gone all along": awarding grants to

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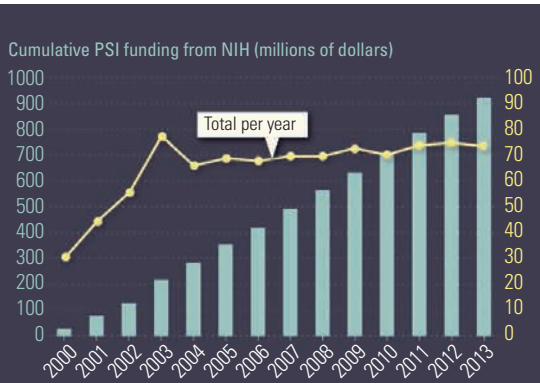

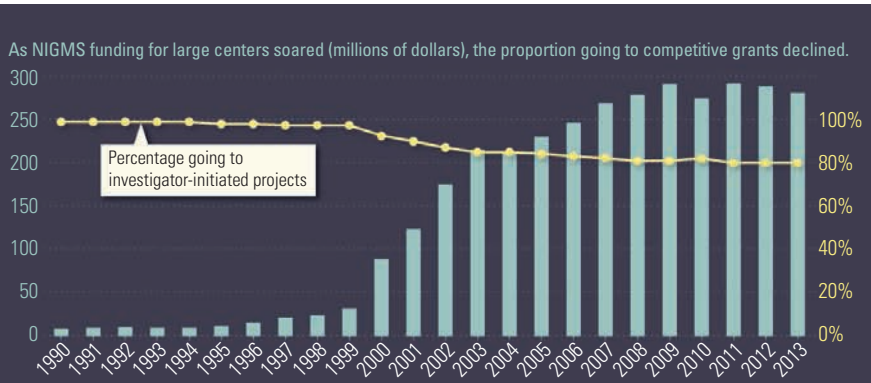

projects deemed most valuable by conventional peer review.

Joachimiak says such criticisms are too facile. According to one estimate, the cost of producing the structure for one of the easier "soluble" bacterial proteins has plunged about 56% since 2003 to about $50,000 per structure. A good chunk of the high-speed robotics and software that PSI labs developed for protein expression, purification, crystal growth, and x-ray structure determination are now in standard use by structural biology labs around the world. According to Helen Berman, an x-ray crystallographer at Rutgers University in Piscataway, New Jersey, who runs both the PDB and a PSI archive known as the Structural Biology Knowledgebase (SBKB), the PSI has produced 421 different technologies that have been either commercialized or disseminated through the SBKB online.

CREDIT: RIGAKU AUTOMATION

Built for speed. The PSI revolutionized a host of technologies, including robotic systems like this one for generating protein crystals en masse.

Beyond technology, Joachimiak and others argue that the PSI has made fundamental contributions to protein science. For example, Ian Wilson, a structural biologist at Scripps, and his colleagues have used the suite of tools at their high-throughput center to determine the structures of a large number of HIV and influenza viral proteins. Their goal is to identify common features in the proteins from each virus, which could provide targets for novel vaccines that would stop a wide variety of viral strains at once, rather than the one or two strains hit by current vaccines. And David Baker, a computational biologist at the University of Washington, Seattle, has used dozens of structures of stripped-down "ideal" proteins solved by the Northeast Structural Genomics Consortium—a PSI effort—to sort out rules for designing novel proteins never made by natural organisms. Baker and colleagues are now using those rules to design synthetic proteins to serve as gene therapy agents, catalysts for converting carbon dioxide into fuel, and a host of other applications.

But Michigan's Smith says projects such as Wilson's HIV work and Baker's protein design would have thrived anyway in a competitive funding environment of individual investigator awards, known as R01 grants. Meanwhile, she says, "there are a lot of problem-based structural biology projects of very high merit that are not getting funded right now, because there is not enough money." If NIGMS redirects some of the money now spent on the PSI into investigator-initiated grants, "this will be positive," she says.

Critics also fault the PSI for failing to identify enough rules of protein folding so that structures can be computed from their sequence, rather than laboriously solved. "There is no doubt that if you have a close [gene] sequence homology then you can do a lot of successful modeling," says Michael Levitt, a computational biologist at Stanford University in California. However, he adds, "protein folding has not yet been solved generally." PSI investigators concede the point. "Our computational methods still aren't strong enough yet," Stevens says. Levitt adds that even though PSI investigators have produced thousands of protein structures, the number of gene sequences encoding unknown proteins has grown much faster, to more than 30 million. As a result, Levitt says, "it would take a very long time and an enormous amount of money" to solve structures of representatives of a large percentage of protein families.

Such brute-force efforts are now off the table, and the current PSI centers will be dismantled over the coming years. "The question is, how can we make this transition as orderly as possible with minimal collateral damage?" says Smith, who serves on the panel of outside experts advising the PSI on how its assets should be distributed. One option is for NIGMS to continue to fund high-throughput protein expression, production, and crystallization facilities as centralized resources for the whole structural biology community to use. Another is to distribute some of these facilities and technologies among current structural biology labs. These high-speed tools "shouldn't just go away," Smith says. NIGMS hopes to decide between May and December of this year, after the panels are expected to submit their recommendations.

PSI investigators say dismantling their centers could imperil U.S. leadership in structural biology. "A lot of jobs will be ending," Stevens says. "We'll see a very significant drop-off in the number of protein structures coming from the U.S." Meanwhile, other countries, notably China, are ramping up their own efforts in high-speed structural biology. "I'm worried," Joachimiak says. "We've made incredible progress. Now we're looking at just shutting it down." Wilson agrees. "We need a balance" between R01-type work and larger scale projects, he says.

But Douglas Sheeley, a program officer at NIGMS who is overseeing the work of the two PSI transition panels, says the PSI's termination does not mean the agency is ending its support for structural biology or the collaborative team-based science that the PSI promoted. In 2012, NIGMS spent $164 million to support structural biology, roughly 70% of the NIH total.

That total will almost certainly go down, because it includes $75 million for the PSI. But Harrison insists that the U.S. structural biology community will thrive without the dedicated funds. NIGMS officials are considering using the PSI's budget to fund an increasing percentage of R01-type grants. Even if that money no longer supports structural biology, "I think that's okay," Harrison says. It will force all structural biology projects to justify their merit against all other research. "We should compete on an even playing field."

–ROBERT F. SERVICE

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