

TIME-RESOLVED MACROMOLECULAR CRYSTALLOGRAPHY

The processes of life depend on an extensive array of chemical transformations within a cell. Sugar must be metabolized into useful chemical energy stores, raw materials acquired from the environment must be converted into a wide array of biochemical reactants, and the basic functions of the cell must be carried out. To regulate these processes and to speed up reactions that are intrinsically slow, every cell has an arsenal of specialized protein molecules. These large molecules—each including thousands of atoms—can be amazing catalysts, increasing reaction rates by as much as 17 orders of magnitude. Protein molecules are heteropolymers consisting of sequences of 20 different amino-acid building blocks that fold up into a well-defined three-dimensional structure. The structure of such large and intricate molecules is a key determinant of their function and effectiveness, and biochemical reactivity is often a consequence of the molecule's physical properties. However, knowledge of structure is not enough to determine how a protein functions, because biological processes typically follow a path consisting of multiple reactions involving sometimes short-lived chemical intermediates. The best picture of protein function can be obtained by watching the protein in action, following its structural changes at different points along the path of a catalyzed reaction and observing it in complex with reactants, products, and any chemical intermediates. Through a variety of techniques collectively referred to as time-resolved x-ray crystallography, researchers are now constructing this dynamic picture.¹

In addition to increasing our understanding of the physical chemistry of catalysis, time-resolved crystallography experiments have many practical applications. For example, studies of protein functioning in disease-causing organisms could lead to ways to fight illness. Understanding the mechanisms by which natural proteins work should aid in designing novel protein catalysts to make industrial processes more efficient (for a good review of biocatalysis, see ref. 2).

Visualizing macromolecular structure

X-ray crystallography is the predominant technique for determining molecular structures at atomic resolution, and has been used for the past 40 years to visualize protein, DNA, and RNA macromolecules² (see the article by

Rapid x-ray characterization of structure and innovative ways of initiating and controlling reactions are shedding new light on protein function by enabling the visualization of macromolecules in action.

Eric A. Galburt and Barry L. Stoddard

Wayne A. Hendrickson in *PHYSICS TODAY*, November 1995, page 42). This method, which is simply a powerful form of microscopy, uses x rays as a source of light and images the macromolecule(s) of interest in crystal form. Soft x rays are used because their wavelength (0.7–1.5 Å) is comparable to the distance between bonded atoms in molecular structures (1.0–1.5 Å), so that atomic positions can, in principle, be resolved in the calculated molecular structure (see the article by Neville Smith in *PHYSICS TODAY*, January 2001, page 29).

A protein crystal contains between 10^{13} and 10^{16} molecules arranged in a precisely defined 3D lattice. The crystal lattice causes the constructive and destructive interference of scattered x rays that results in a diffraction pattern according to Bragg's Law, with the geometry of the diffraction pattern determined by the symmetry of the crystal lattice. The amplitudes of the individual reflections that make up the 3D diffraction pattern are determined by a variety of experimental and computational techniques, resulting in electron density contour maps that show the structure of the fundamental building block (or asymmetric unit) of the crystal (see the article by Jerome Karle in *PHYSICS TODAY*, June 1989, page 22). On this basis, investigators can build a detailed model and construct a single, static image of the molecule.

A typical laboratory setup is shown in figure 1. For macromolecular crystallography, the most common monochromatic x-ray source is the copper K α line (1.54-Å wavelength) generated by directing an electron beam onto a rotating Cu anode. Alternatively, synchrotron radiation generates a higher flux with a tuneable x-ray wavelength³ (see the article by Sebastian Doniach in *PHYSICS TODAY*, April 1985, page 68).

Proteins are crystallized for structure determination, but most protein activity of interest naturally occurs in solution (for example, inside a cell), rather than in the crystal state. Nevertheless, the same reactions can usually be made to occur both in solution and in the crystal, though possibly at different rates. Between 40% and 60% of the volume of most protein and nucleic-acid crystals is composed of solvent, in the form of water found in large channels and ordered hydration shells around the macromolecules. As a result, small reactant molecules (which are usually less than 100 atoms in size) can diffuse throughout a crystal of an enzyme (a protein that catalyzes a specific chemical reaction), bind to its active site (the reactive center of the enzyme), and be turned over (undergo a catalyzed transformation). Noncatalytic processes, such as binding reactions, conformational

ERIC GALBURT is a graduate student and BARRY STODDARD is a professor at the Fred Hutchinson Cancer Research Center in Seattle, Washington and the graduate program in biomolecular structure and design at the University of Washington.



FIGURE 1. TYPICAL INSTRUMENTATION used for measuring x-ray diffraction of macromolecular crystals. The large image shows two x-ray detectors (large boxes on the left and right of the image) and two goniometers. The inset images are progressive closeups of the setup. The left image shows the goniometer on which the crystal is positioned. The x-ray collimator (horizontal, pointing left), cooling nozzle (vertical, pointing down), and centering microscope (horizontal, pointing back) can all be seen. The black piece of metal affixed to the collimator and around the cooling nozzle is a beam stop that prevents the majority of x-rays that pass directly through the crystal from saturating the detector. The crystal is harvested from its growth environment with the fiber loop shown in the center image and plunged into liquid nitrogen for rapid cooling. The loop is then placed on the goniometer and positioned so that the crystal is centered in the x-ray beam. The rightmost image shows a typical protein crystal. (Courtesy of Brett Chevalier.)

(shape) changes, and electron transfer steps, can also be induced in crystals.

Reactions in the crystalline environment may exhibit kinetic rates similar to those measured in solution, or may be significantly slower due to restraints imposed by the crystal lattice. In either case, it is often possible to accumulate and collect x-ray diffraction data for a rate-limited intermediate species (one for which net production rates exceed net decay rates) within its lifetime. A key reason that such experiments are possible is that efficient methods have been developed over the past 15 years that allow x-ray diffraction data to be collected at extremely rapid speed. When data are collected using the polychromatic Laue diffraction method at a modern synchrotron x-ray source, exposure times can be as short as 10–100 picoseconds and still provide useful signal-to-noise ratios⁴ (see the box on page 35).

Time-resolved techniques

For time-resolved experiments, the investigator must address three critical issues. First, the reaction must be initiated in such a way that an intermediate species accumulates uniformly throughout the crystal. Next, if the desired intermediate is not inherently rate limited or displays a lifetime too short to allow its visualization, then a strategy is usually adopted to stabilize or “trap” that intermediate. Finally, an appropriate method of x-ray data collection

must be chosen, sufficiently rapid to match the lifetime of both the transient reaction intermediate and the crystal specimen itself.

The choice of experimental strategies for time-resolved crystallographic experiments depends on the kinetics of the reaction, the conformational changes exhibited by the molecule during the reaction, and the size, symmetry, stability, and solvent content of the crystals.⁵ At the fastest end of the time-resolved experimental spectrum is a so-called single-turnover experiment, in which all the data are collected during a single reaction event. This method is usually chosen either because the reaction can only occur once (the reaction does not cycle repeatedly and is also not reversible) or because after the initial reaction event, the intermediate accumulates nonuniformly or not at all. In single-turnover experiments, the reaction is usually triggered by a photolytic event, and the resulting accumulation and decay of the desired intermediate species occurs on a nanosecond-to-millisecond timescale. At the slowest experimental extreme, reactions can be initiated by slow diffusion of substrate into the

crystal. In this type of protocol, the intermediate is often trapped using chemical and physical techniques such as reduced temperature or the use of a nonoptimal solvent or pH for the reaction.

The simplest method of reaction initiation is to diffuse a reactant species into the crystal. This method can be used either to trigger a single reaction event or to continuously drive a reaction cycle. For the latter strategy—often called a steady-state experiment—the reaction is driven in the crystal using a continuous flow of a high concentration of reactants to guarantee that the majority of the reaction sites in the crystal are continuously engaged in the reaction. Both strategies have been used to study a wide variety of molecular reactions. For example, experiments based on collecting all the diffraction data during a single reaction event have determined the mechanism of self-cleavage of the hammerhead ribozyme (discussed later); and Janos Hajdu’s group at Uppsala University has used this strategy to study reactions that oxidize and reduce small molecule substrates, such as the reactions catalyzed by cytochrome C peroxidase and catalase. Steady-state experiments have been used to visualize intermediates in the catalyzed hydroxylation of camphor by the enzyme P450cam (discussed later) and in the rapid metabolic transformations of isocitrate dehydrogenase that lead to the formation of alpha-ketoglutarate, a reaction that is part of the Krebs cycle.⁶

The Laue Method and Synchrotron Radiation

In November 1895, Wilhelm Conrad Röntgen conducted a set of experiments that would lead to his receiving the first Nobel Prize for Physics. His observations were on the properties of a novel form of electromagnetic radiation, produced as a byproduct of a cathode-ray generator. These emissions were termed “x” rays, to denote that their characteristics were unknown (see the special issue of *PHYSICS TODAY*, “X Rays 100 Years Later,” November 1995). Röntgen demonstrated that the rays had no electrical charge, were generated at the point of contact of the cathode rays with the interior of the apparatus tube, and could pass through relatively long distances of air, as well as paper and soft animal tissue.

For years after his discovery, the basic nature of x rays was debated. Proponents of models describing x rays as nonperiodic packets of energy vied with those favoring electromagnetic waves. The debate was effectively resolved in 1912 when Max T. F. von Laue and two students (Walter Friedrich and Paul Knipping) demonstrated the wave nature of x rays and the periodic nature of crystals by observing the diffraction of x rays from crystals of zinc sulfide. Their studies were the first example of the application of x rays for crystallographic analyses.

The experiments performed by von Laue were, by necessity, carried out using a sealed tube generator producing a low-energy, polychromatic, bremsstrahlung x-ray continuum. To this day, the use of polychromatic x rays for molecular structure determination is known as the Laue method. In the decades after these crucial studies, several experimental issues conspired to prevent this method from being used extensively for crystal structure determinations. Most important, a significant fraction of polychromatic data contain multiple orders of diffracted x rays, called harmonic overlaps, that were generally considered to be difficult to deconvolute. In addition, the intensities of the diffracted x rays vary as a function of the incident spectrum and must therefore be normalized. Finally, polychromatic diffraction patterns from macromolecular crystals are prone to spatial overlaps due to the large number of reflections recorded on individual exposures. Therefore, the method of choice for x-ray structure determination has been to use monochromatic x rays and to collect diffraction intensities in a series of exposures as the crystal is rotated through a wide arc. This is an inherently slow method,

taking from minutes to days to collect a complete data set depending on the strength of the x-ray beam.

In the late 1980s, as investigators realized that polychromatic diffraction data could be collected at x-ray synchrotrons using very short exposure times, interest in the Laue method increased. Synchrotrons produce extremely intense x-ray beams (between 10^{11} and 10^{14} photons $\text{sec}^{-1} \text{ mrad}^{-1}$), with a featureless polychromatic spectral profile. The precise nature of the spectrum and flux produced at any experimental station at the synchrotron can be controlled by the use of magnetic insertion devices that create local fluctuations in the orbital radius of the electron current. Although most crystallographic studies at synchrotrons are performed somewhat rapidly using monochromatized x-ray beams—in essence throwing out the majority of the available x-ray flux—it is possible to use x-ray beams with broad spectral profiles (typically spanning wavelengths from 0.7 to 1.5 Å) for diffraction studies.

The use of a polychromatic x-ray beam actually allows a large fraction of all potential diffracted x rays within a data set to be measured from a single crystal orientation. An example of this can be seen in the illustration on the cover, which simulates a polychromatic Laue x-ray diffraction pattern produced at the National Synchrotron Light Source at Brookhaven National Laboratory from an isocitrate dehydrogenase crystal. Such an image, taken using a single crystal orientation, records approximately 45% of a complete data set; to collect an equivalent amount of data by monochromatic x-ray methods, the crystal would have to be rotated through roughly 22° , requiring approximately 30 separate exposures at 0.7° per image. This feature of the Laue method, combined with the enormous x-ray flux available at the most powerful synchrotron facilities, enhances the rate of data collection into time regimes approaching the lifetimes of even the most transient of biological reaction intermediates.

Over the past 15 years, since the first Laue diffraction patterns were collected from macromolecular crystals, many of the difficulties in measuring and deconvoluting polychromatic diffracted x-ray intensities have been overcome through the use of straightforward computational algorithms. It is now possible to collect useful diffraction data from protein and nucleic acid crystals at almost any rate that is desired.

Regardless of the exact nature of the experiment, diffusion is far too slow for characterization of anything other than exceptionally long-lived intermediates. For observations based on a single reaction event, uniform accumulation of a unique rate-limited species throughout the crystal can be achieved only for systems with overall rates (from initial substrate to final product) of 10^{-2} reactions per second per molecule. For a steady-state experiment, the reaction rate that can be successfully accommodated is somewhat higher, up to approximately one reaction per second per molecule.

Many biochemical reactions must be initiated using faster methods than diffusion. The use of photoreactive “caged” compounds has enabled investigators to trigger the rapid release of biologically important effectors. Caged compounds are reactive molecules that have been deactivated by slightly changing their structure or composition in such a way that the original reactivity can be restored by exposure to light (typically in the visible or ultraviolet region). A reactive substrate molecule can be diffused into an enzyme crystal in caged form and then be rapidly light-activated. The timing of the activating signal—often from a laser—is coordinated with that of the x-ray exposure to optimize the quantity and distribution of

the desired intermediate in the crystal during imaging. In recent years, extraordinarily efficient photoreactive caging groups have been created that can be incorporated onto hydroxyl, carboxyl, phosphoryl, and amide groups of a variety of compounds. Almost all are capable of submicrosecond product release.

Photolysis can also form the basis for extremely rapid x-ray observations in studies of intermediates formed by photoreaction centers. These molecules possess the inherent advantage of having the photoexcitable molecule (a reactive chromophore) bound to the protein or nucleic acid at virtually 100% occupancy before reaction initiation. Other advantages are the reactant molecule's high photochemical efficiency and the well-characterized, separable spectroscopic signals often exhibited by each reaction intermediate. Nanosecond time resolution can be achieved for early events in these reactions, far faster than the resolution achievable for any system that requires a binding event after the initial triggering step.

Electron crystallography has been used for imaging in time-resolved diffraction experiments that use photolysis triggering, particularly with membrane-bound receptor proteins (for related work on electron cryomicroscopy of proteins, see *PHYSICS TODAY*, March 1999, page 21).

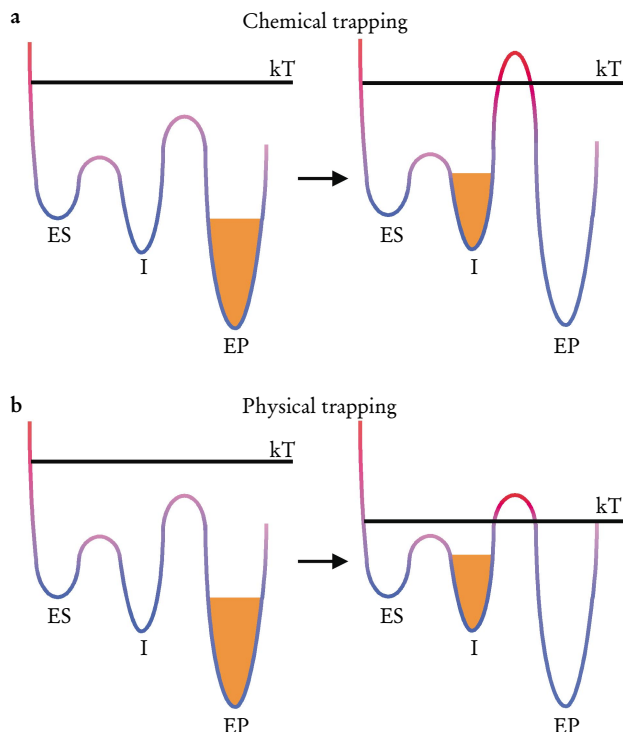


FIGURE 2. CHEMICAL AND PHYSICAL TRAPPING are illustrated using hypothetical free-energy diagrams. The y -axis represents energy (with the ambient temperature kT indicated by the thick horizontal line) and the x -axis represents the reaction coordinate. The (identical) left diagrams indicate the unperturbed energy landscape of the catalyzed reaction and the right diagrams illustrate the effect of trapping. The three energy minima correspond to the three states in the simplified reaction: the enzyme-substrate complex (ES), a stable intermediate (I), and the enzyme-product complex (EP); orange shading indicates which state is populated under the given conditions. In the unperturbed case, EP is populated because there is enough thermal energy to push the system over both of the energy barriers, but the effect of trapping is to make the EP state inaccessible and so populate the I state. (a) In chemical trapping, kT remains constant while the energetics of the reaction are modified so that the system cannot get over the second barrier. (b) In physical trapping, the energy landscape remains constant while cooling causes kT to decrease so that there is not enough thermal energy to proceed over the second barrier.

energy for the system. For example, for a reaction step with an activation energy barrier of 4 kcal/mol, a temperature reduction from 20° C to -80° C will reduce the rate by a factor of $1/30$; for a reaction step with an energy barrier of 10 kcal/mol, the same temperature reduction will reduce the rate by more than three orders of magnitude.

Destruction of the sample by the formation of ice can generally be avoided by transferring the crystals before cooling to mixed solvent systems (cryosolvents) with a high viscosity and depressed freezing point. However, other difficulties can arise as a result of very low temperatures. The physical constants of some chemical groups in enzyme catalysts show a large and variable temperature dependence. For example, in the ionizable groups that are often involved as critical residues in catalysis, the pK_a value (a measure of the propensity to accept a proton) varies with temperature to a degree that depends on the enthalpy of ionization for the group. Thus, cooling could alter the catalytic mechanism in an unexpected manner. Other complications may arise from a change in the dielectric constant of the reaction medium with cooling or from the change in composition of the medium when cryosolvents are used.

Instead of trapping intermediates by conducting a reaction at a low temperature, experimenters could initiate the reaction at room temperature and then trap the desired intermediate by extremely rapid cooling. Methods of flash freezing, now commonly used to immortalize crystals for data collection, can also be applied for intermediate trapping experiments. In theory at least, this method makes the reaction profile less susceptible to the problems discussed earlier; the only possible confounding factor is the presence of cryoprotective agents such as glycerol or sucrose.

Chemical trapping may also be used, either separately or in conjunction with low-temperature and other physical trapping strategies. For this strategy, the chemical nature of reaction components is adjusted so that a particular intermediate accumulates in the crystal; in essence, the kinetic profile of the catalytic reaction is altered to impose a novel rate limit or a thermodynamic dead end. Chemical trapping may be accomplished by significantly changing the pH or by perturbing the solvent environment of the reaction. With single-substrate/single-product reactions (or those that proceed through separable half-reactions), conditions of thermodynamic equilibrium can be adjusted to favor a single predominant catalytic

Studies of bacteriorhodopsin by Richard Henderson of the Medical Research Council Laboratory of Medical Biology in Cambridge, UK, and of the acetylcholine receptor by Nigel Unwinand, also of MRC, have allowed the direct visualization of transient conformational and reaction intermediates formed by these proteins. These experiments are greatly facilitated by the two-dimensional character of electron diffraction experiments, in which the protein is arrayed as a crystalline monolayer across a sample grid. This allows rapid and efficient reaction triggering either by flash photolysis (used for bacteriorhodopsin) or by spray-mixing ligand on the receptor molecules followed by flash cooling of the sample. Because the sample thickness is approximately the width of a single unit cell, the efficiency of reaction initiation and of cooling and trapping is greater than in 3D crystals.

Stabilization and trapping

The techniques used to extend the lifetime of an intermediate species fall into two broad, overlapping categories: physical trapping and chemical trapping (see figure 2). Both methods can be used to reduce the rate of one or more steps in a biological reaction, allowing the use of the simplest method of x-ray data collection and maximizing the signal-to-noise ratio through longer exposure times. In systems with well-characterized reaction pathways and several intermediates, each predominant at different temperatures, exploiting unique trapping protocols allows crystallographers to perform detailed mechanistic studies.

The use of low temperatures has become an increasingly versatile tool for time-resolved studies. By varying reaction conditions, cooling rates, and final temperatures in separate experimental runs, different and sometimes closely related reaction intermediates can be trapped. The ability to use a reduction in temperature to favor a particular reactant species stems from the different temperature dependences of different reaction pathways. The rate constant for a single kinetic step in a reaction typically declines with decreasing temperature, with the magnitude of the decline dependent on the absolute activation

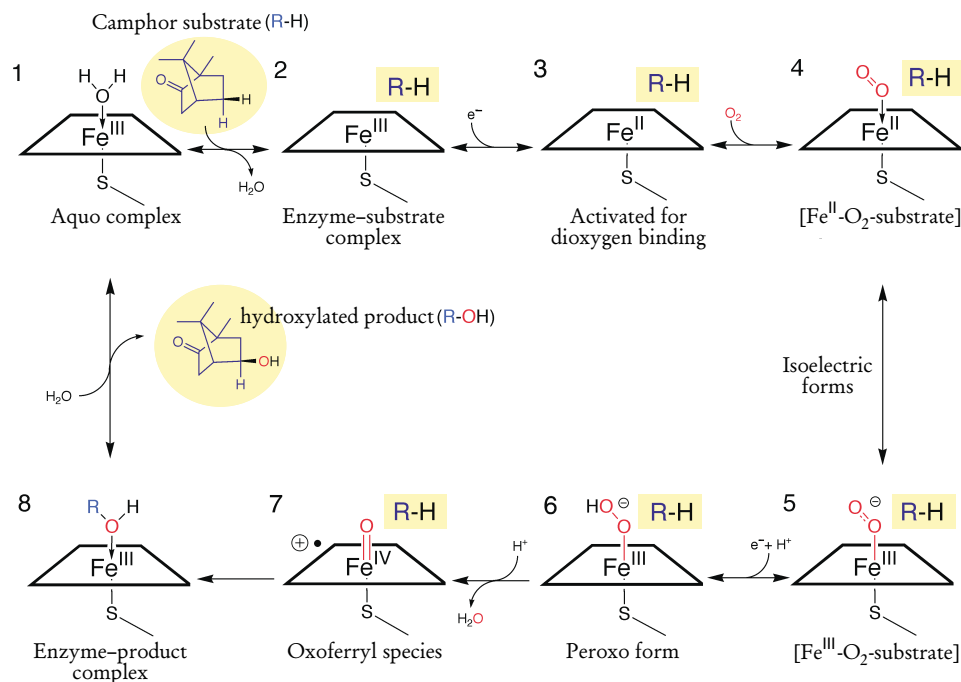


FIGURE 3. REACTION CYCLE OF CYTOCHROME P450CAM as it catalyzes the oxygenation of camphor to produce 5-hydroxycamphor. The enzyme makes use of a bound heme molecule at the active site, which is represented schematically by the trapezoidal box surrounding the iron atom; as the reaction progresses, the iron atom alternates between three charge states: ferric iron (Fe^{III}), ferrous iron (Fe^{II}), and a short-lived oxoferyl species (charge state Fe^{IV}). Steps in the reaction cycle are numbered sequentially. The reaction cycle begins with the enzyme binding to the camphor substrate, represented by the blue diagram pictured at step 1 and denoted in subsequent steps by **R-H**. When the enzyme binds to camphor, a metal-bound water molecule is displaced and its site is then occupied by an oxygen molecule (step 4) that ultimately serves as the source of the new oxygen atom in the final product. After a number of intermediates, the cycle arrives at the enzyme-product complex (step 8), which, with the addition of water, yields the hydroxylated product and restores the enzyme to its initial form (step 1). Experimental structures of intermediates 3, 6, and 7 in this pathway have recently been reported by Schlichting and coworkers using a variety of time-resolved strategies.⁹

ic species. More sophisticated methods of chemical trapping use site-directed mutagenesis, chemical modification of the substrate, or a cofactor (a substance whose presence is required for enzyme activity) to extend the lifetime of the catalytic intermediate of interest.

Caught in the act

Time-resolved crystallographic studies have been particularly productive for nuclease and phosphatase enzymes, ribozymes (RNA macromolecules that catalyze chemical reactions, particularly making and breaking phosphodiester bonds), electron transport proteins, photoreactive proteins, and a variety of metabolic enzymes.

Some of the most ambitious time-resolved studies to date are those of photoreactive yellow protein (PYP) by Elizabeth Getzoff's group at The Scripps Research Institute and Keith Moffat's group at the University of Chicago. This photolysis-based work has produced snapshots of several intermediates formed by PYP and its associated chromophore during their photocycle reaction. PYP acts as a light receptor that initiates a phototactic response (a movement toward light of specific wavelengths) in halophilic bacteria. After the reaction is triggered, the protein undergoes a series of transformations. The triggering photon is absorbed by a bound chromophore (a light-sensitive molecule), producing an electronically excited P^* state that rapidly

changes its shape to become intermediate I_1 . A protein rearrangement then occurs, converting I_1 into the longer-lived (several-second lifetime) intermediate I_2 . The structure of the enzyme ground state has been determined by traditional crystallographic methods and that of the longest-lived intermediate (I_2) was determined by single-turnover Laue studies using repeated light-pumping on a millisecond timescale.⁷ Recently, the structures of two early, short-lived (nanosecond lifetime) intermediates have been determined by using both low-temperature trapping and a combination of rapid phototriggering and Laue diffraction at ambient temperatures.⁸ These studies are remarkable for their success in obtaining high-resolution maps of the structures of different, closely related species on a photochemical reaction pathway, in some cases using rapid diffraction techniques to obtain nanosecond time resolution.

Photolytic reaction initiation has also been used by other investigators, including Joel Berendzen at Los Alamos National Laboratory, Ilme Schlichting at the Max Planck Institute for Molecular Physiology in Dortmund, Germany, and the Moffat

group, to visualize the photodissociation and rebinding of carbon monoxide to myoglobin.

In an especially impressive recent study, Schlichting, Greg Petsko at Brandeis University, and collaborators used a steady-state protocol to determine the structures of a series of intermediates formed by the cytochrome P450cam protein as it catalyzes the oxygenation of a camphor molecule⁹ (figure 3). This enzyme is part of a family of molecules (humans have more than 100 different kinds of P450 enzyme) that are responsible for detoxification of foreign compounds. P450cam follows a complex reaction cycle in which a protein-bound metal in the enzyme active site oscillates through three separate oxidation states. Time-resolved experiments revealed structures for several key intermediates on this pathway by using a combination of trapping techniques, including low temperatures, and by driving the reaction in frozen crystals using electrons generated by radiolysis in the x-ray beam at the synchrotron.

Three additional examples illustrate the capabilities of the technique and range of the field. In all of these studies, one or more structures were determined of at least one reaction species that is normally shortlived. In each case, knowing the structures of reactants enabled researchers to see which protein groups interact with which reactant groups during different phases of the reaction. The time-resolved strategies used ranged from extremely slow or

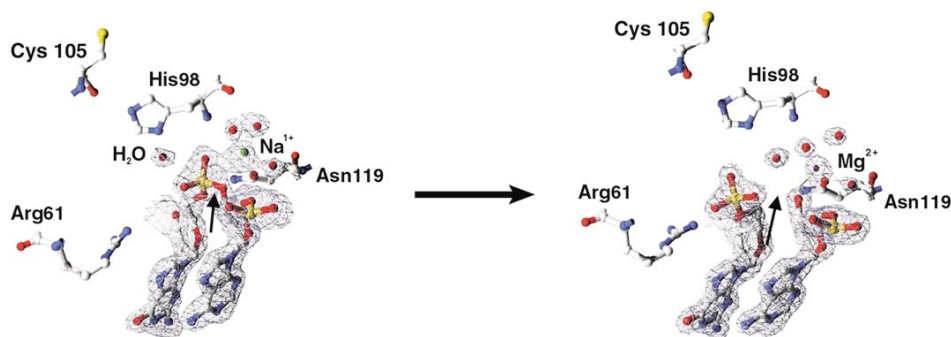


FIGURE 4. CLEAVAGE OF A DNA PHOSPHODIESTER BOND by the protein enzyme I-PpoI, as visualized by so-called static time-resolved crystallography. (The experiments are called static because pre-trapped species were crystallized independently, rather than initiating and trapping a reaction after crystallization.) The structure on the left represents an intact, trapped complex prior to the reaction, and the structure on the right represents the cleaved product. An arrow indicates the bond that breaks in the reaction. The fine gray mesh of lines indicates the experimental electron density contour map; this is overlaid by colored lines denoting the modeled positions of atoms, with the color denoting atomic species (for example, carbon is white, oxygen is red, and nitrogen is blue). The amino acids making up the active site are labeled with their sequence number on the linear protein polymer. The uncleaved complex was trapped chemically by substituting a sodium ion (Na^{+}) for a bound magnesium ion (Mg^{2+}); when the Mg ion is restored, as shown on the right, the reaction occurs.

static (the catalyzed breaking of a DNA bond by I-PpoI, illustrated in figure 4, where the reaction substrate complex was trapped before crystallization¹⁰) to moderately slow (the self-cleavage of the hammerhead RNA enzyme,¹¹ shown in figure 5, where the reaction is slow enough that diffusion triggering and single-reaction methods could be used) to fast (the metabolic transformation of isocitrate dehydrogenase mentioned previously, for which steady-state and flash-photolysis methods were used). For more examples, including several metalloenzymes and the proton pump bacteriorhodopsin, see reference 12.

Future directions

A particularly exciting application of time-resolved crystallography is the ability to directly correlate chemical or structural perturbations in a reactive site with corresponding

constraints for various theoretical strategies, such as free energy perturbation simulations and nudged elastic band (NEB) calculations.

Computer simulations based on molecular dynamics have been used in macromolecule studies for at least two decades, but significant progress is only now being made, largely because of the increase in the number of intermediate structures that are known to high resolution. Simulations involving perturbations in the free energy of the system and using a semiempirical potential have recently been used by Arie Warshel and coworkers at the University of Southern California to study the staphylococcal nuclease-catalyzed reaction, the initial proton transfer step in human carbonic anhydrase I, and guanosine triphosphate hydrolysis by ras p21.¹³

The NEB method exploits the additional constraints given by known intermediate structures to determine the minimum-energy path of a given reaction.¹⁴ In contrast to free-energy perturbation techniques, where the reaction coordinate is controlled by dragging the components of a

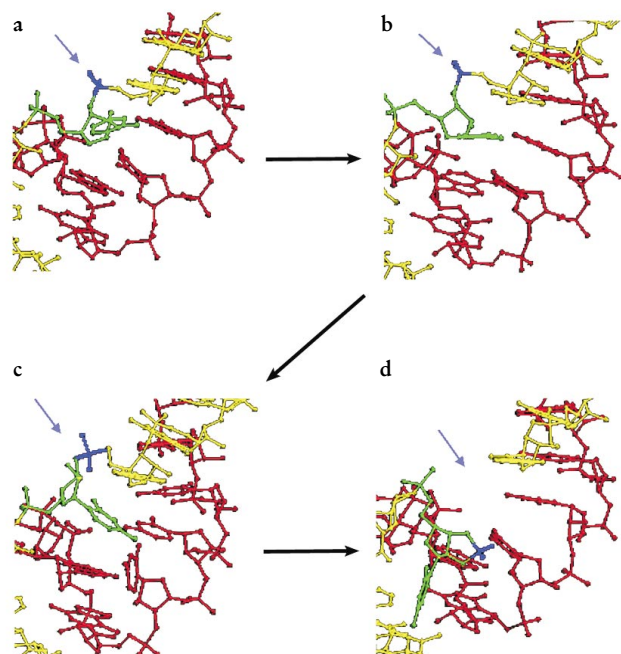


FIGURE 5. A SERIES OF ATOMIC-MODEL SNAPSHOTS of the hammerhead RNA enzyme, or ribozyme, as it cleaves itself at one specific phosphate group (blue atoms); only a small part of the full enzyme is shown. These time-resolved observations were made during a single reaction event in the crystal, using both chemical and physical trapping. The reaction was triggered by diffusing a jump in pH into the crystal. The ribozyme consists of two RNA strands, colored red and yellow. The cleavage point is indicated by the blue arrow, and the large black arrows denote the progress of the reaction. (a) Ground state before the reaction. (b) Conformational intermediate produced before cleavage and preserved by freeze-trapping. (c) A second conformational intermediate, trapped by chemical modification of the ribozyme. (d) The cleaved product complex. Note the significant conformational change exhibited by the target phosphate group and by the neighboring nucleotide base (colored dark green). A movie of this reaction, based on these structures, is available at <http://chemistry.ucsc.edu/%7Ewgscott/pubs/movies.html>.

simulation toward a previously defined product state, the NEB method defines the reaction coordinate based on an underlying energy landscape, which can be supplied by any number of different potentials, ranging from empirically parameterized classical potentials to quantum-mechanical ones. The reaction coordinate is defined by minimizing the energy of a chain of structures that are inserted between the stable intermediates. Elaborations of this technique have been recently used by Hannes Jónsson at the University of Washington in studies of atomic motion on 2D surfaces¹⁵ and of the growth of both crystalline and amorphous solids.

The time resolution afforded by rapid diffraction methods is now as fast as 10–100 ps, which is adequate for almost any crystallographic studies of macromolecular structure. However, recent research has begun to address the possibility of probing reaction processes at time resolutions three orders of magnitude faster, which is fast enough to resolve and study atomic vibrations. X-ray pulses as short as 100 femtoseconds have been generated at the Advanced Light Source at Lawrence Berkeley National Laboratory. Recent developments in free-electron lasers (FELs) could revolutionize both the timescales at which macromolecular structure data are obtained and the geometrical requirements for data acquisition. Work by Hajdu and coworkers has shown the possibility that single-molecule diffraction, based on short, high-flux x-ray bursts from an FEL, could be used to generate macromolecular structural information.¹⁶ Significantly, this technique would remove the requirement of crystallizing the sample of interest. Major experimental hurdles remain, including optimizing the sample position in the beam and preventing the extremely large amount of radiation from destroying the sample. However, these innovative experimental techniques will greatly increase the timescales available to future crystallographers interested in studying how macromolecular systems change in time.

References

1. *Time-resolved Macromolecular Crystallography*, D. W. J. Cruickshank, J. R. Helliwell, L. N. Johnson, eds., Oxford U. Press, New York (1992).
2. C. Walsh, *Nature* **409**, 226 (2001) and the reviews that follow in that issue.
3. M. Altarelli, F. Schlachter, J. Cross, *Sci. Am.* December 1998, p. 66.
4. K. Moffat, in *Methods in Enzymology*, vol. 277, C. Carter, R. M. Sweet, eds., Academic Press, San Diego, Calif. (1997), p. 433. Z. Ren et al., *J. Synchrotron Radiat.* **6**, 891 (1999).
5. I. Schlichting, R. S. Goody, in *Methods in Enzymology*, vol. 277, C. Carter, R. M. Sweet, eds., Academic Press, San Diego, Calif. (1997), p. 467.
6. J. M. Bolduc et al., *Science* **268**, 1312 (1995).
7. U. K. Genick et al., *Science* **275**, 1471 (1997).
8. U. K. Genick et al., *Nature* **392**, 206 (1998). B. Perman, *Science* **279**, 1946 (1998).
9. I. Schlichting et al., *Science* **287**, 1615 (2000).
10. E. A. Galburt et al., *Nat. Struct. Biol.* **6**, 1096 (1999).
11. J. B. Murray, D. P. Terwey, L. Maloney, A. Karpeisky, N. Usman, L. Beigelman, W. G. Scott, *Cell* **92**, 665 (1998).
12. J. Hajdu et al., *Nat. Struct. Biol.* **7**, 1006 (2000).
13. J. Aqvist, A. Warshel, *J. Mol. Biol.* **224**, 7 (1992). T. Schweins, R. Langen, A. Warshel, *Nat. Struct. Biol.* **1**, 476 (1994).
14. G. Henkelman, G. Johansson, H. Jónsson, in *Progress on Theoretical Chemistry and Physics*, S. D. Schwartz, ed., Kluwer Academic (2000), p. 269.
15. G. Henkelman, H. Jónsson, *Phys. Rev. Lett.* **86**, 664 (2001).
16. R. Neutze, R. Wouts, D. van der Spoel, E. Weckert, J. Hajdu, *Nature* **406**, 752 (2000). ■

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