Time-Resolved Crystallography

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Abstract

Time-resolved crystallography has been successfully applied on the time scale from seconds *via* milliseconds and nanoseconds to picoseconds on a variety of systems. This brief review largely deals with macromolecular systems, on which there has been substantial recent progress. The strategies for design of a successful experiment that eliminates or minimizes potential artefacts have been identified, and the specifically crystallographic components of these strategies have been implemented. The remaining computational challenge is to identify and extract time-independent structures, each corresponding to a distinct reaction intermediate, whose populations vary with time and give rise to the time-dependent X-ray diffraction data. The fourth dimension, time, has been added to the three spatial dimensions of crystallography; it can no longer be regarded as purely a static technique.

1. Introduction

The essence of a crystal is perfect translational symmetry, which generates structurally identical molecules arranged in a perfect lattice. However, all biological and chemical reactions involve structural change that evolves in time along a reaction coordinate from reactant *via* intermediates to product. Reaction mechanism involves structural change, not just static

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structure. The title 'time-resolved crystallography' thus seems at first glance paradoxical: how can crystallographic techniques be applied effectively to a spatially heterogeneous time-dependent set of structures that do not exhibit perfect translational symmetry (Moffat, 1998)? Crystallography, whether static or time-resolved, can reveal only the space-average structure over all molecules in the crystal and the time-average structure over the duration of the X-ray exposure that generates each individual diffraction image (reviewed by Moffat, 1989; Cruickshank *et al.*, 1992; Hajdu & Johnson, 1993; Helliwell & Rentzepis, 1997). The dynamic aspects of changes in structure during chemical processes or during key biological processes such as enzyme catalysis, binding and release of drugs from receptors or of small molecules from macromolecules, photocycling in light-sensitive systems, and protein unfolding and refolding are critical. However, the rates of structural change can be very large and the lifetimes of transient structures can be correspondingly very short, from femtoseconds (Martin & Vos, 1992) to seconds or, exceptionally, days (Zegers *et al.*, 1998). Thus, static structure is easier to determine than reaction mechanism. If time-resolved crystallography is to examine structural change and mechanism effectively, it must involve very short X-ray exposures, less than the lifetimes of the transient structures that are to be examined. That is, our desired 'molecular movies' must run very fast (Contie, 1997).

2. Time-resolved macromolecular and chemical crystallography

Although I concentrate here on time-resolved macromolecular crystallography, time-resolved small-molecule chemical crystallography may also be conducted (see for example White *et al.*, 1994; Carducci *et al.*, 1997; Ozawa *et al.*, 1998). There are several key experimental differences between the two areas. Macromolecular crystals contain a large volume of liquid and the intermolecular interactions that stabilize the crystal lattice are limited in extent and weak. The environment of the molecules in a macromolecular crystal more resembles that in a concentrated solution. Thus, conformational changes that accompany biological processes in the crystal lattice often proceed *via* the same chemical pathway as in dilute

solution, with similar rate constants, and with retention of the original crystal lattice throughout. In contrast, crystals of small organic and inorganic molecules generally do not contain liquid and the stabilizing interactions are numerous and strong. Conformational changes are greatly restricted, reactivity in the solid state differs qualitatively from that in dilute solution, and reaction is often accompanied by loss of crystallinity and/or a phase change. As a rule of thumb, the fewer the number of atoms involved in a conformational change, the more rapidly it occurs. Time-resolved studies in chemical crystallography and materials science may therefore require the very highest time resolution.

At a more practical level, initiation of a structural reaction in the molecules in a crystal deposits energy in the crystal and this leads to a temperature rise that may itself influence the nature and rate of the conformational change and at worst disorder the crystal. That is, all experiments are unavoidably temperature-jump experiments. This effect can be quite large and differs significantly in magnitude between crystals of macromolecules and small molecules, as I now show (Moffat, 1995).

Consider a structural reaction that is initiated by light. Assume that a crystal contains N molecules, the energy of each photon absorbed is E, a fraction f of that energy appears as heat, and the quantum yield to initiate the photochemical and structural process is Q. Then, since each molecule must absorb one photon, the minimum energy that appears as heat is

$$E_{min} = ENf/Q.$$

Assume further that the concentration of molecules in the crystal is c and that the crystal has a density r and specific heat C_v. Then the minimum adiabatic temperature rise of the crystal is

$$\Delta T_{min} = Efc/QrC_v,$$

which is independent of the crystal volume and proportional to the concentration c of molecules in the crystal. For definiteness, let c be in molar units and set $E = 4 \times 10^{-19}$ J corresponding to 500 nm and $rC_v = 5.6$ J cm^{-3} deg^{-1}. Then,

$$\Delta T \ (^{\circ}C) = 43fc/Q.$$

For macromolecules, the value of c might range from 5 mM (a typical concentration of active sites in a crystal of a relatively large protein) to 100 mM (corresponding to a several-fold excess of a caged reactant over the active site concentration). A value of f/Q of 1.0 is not unrealistic with values of Q in the range 0.1 to 0.65 for many compounds. Thus the crystal temperature rise lies between 0.2 and 4.3°C, even when the absolute minimum number of photons required to fully stimulate the reaction is absorbed. This explains why it is essential to carry out an 'energy titration' to deliver only the minimum number of photons to the crystal (Ng et al., 1995).

Although this calculation is for the minimum adiabatic temperature rise, the rate of heat transfer from the interior of a crystal to its surface from which the heat is dissipated can be quite slow. Thermal equilibration times are milliseconds or longer for typical crystals whose dimensions exceed 50 μm (Moffat et al., 1992; Teng & Moffat, 1998). Thus, in most cases, the experiment is indeed adiabatic; the maximum temperature rise cannot be diminished by more effective cooling.

Further, the dependence of ΔT_{min} on concentration emphasizes the much larger temperature jump for crystals of small molecules. The value of c for such crystals can easily attain 1–50 M, two or three orders of magnitude greater than for crystals of macromolecules. To avoid temperature jumps of several hundred degrees, only a very small fraction of the molecules in the crystal can be allowed to absorb a photon (White et al., 1994; Ozawa et al., 1998). Consequently, stroboscopic or other repetitive signal-averaging techniques must be applied to measure the very small changes in the X-ray diffraction amplitudes accurately.

From the opposite point of view, it is experimentally straightforward to induce a very large temperature jump in such crystals. It is therefore no surprise that the pioneering nanosecond time-resolved crystallographic experiments (Larson et al., 1982) involved laser-induced melting and subsequent recrystallization of the surface of silicon crystals.

A time-resolved crystallographic experiment has five main components. It must be possible: to initiate a structural reaction in a crystal rapidly, uniformly and reproducibly (Moffat, 1989; Ng et al., 1995; Schlichting & Goody, 1997; Stoddard et al., 1998); to monitor the progress of the reaction through the time dependence of the X-ray diffraction pattern of the crystal, using either polychromatic X-rays and a stationary crystal to generate a Laue diffraction pattern (Moffat, 1997) or monochromatic X-rays and a rotating crystal to generate an oscillation pattern (Wulff et al., 1997); to monitor the progress of the reaction in parallel with the X-ray measurements by another technique such as optical absorption (Hadfield & Hajdu, 1993; Chen et al., 1994; Ng et al., 1995); to analyze the time-dependent X-ray diffraction intensities and extract accurate structure amplitudes (J. R. Helliwell et al., 1989; Ren et al., 1996; Clifton et al., 1997); and to interpret in structural terms the resultant time-dependent electron-density maps or difference-electron-density maps.

Here, a major difference between our desired 'molecular movies' and conventional movies becomes apparent. 'Molecular movies' reveal the time-dependent space-average structure in the crystal and this average arises from the superposition of time-dependent populations of time-independent structures. Each time-independent structure corresponds to a significantly

populated intermediate, which is in turn represented by a valley in a plot of the free energy of the system *versus* the reaction coordinate. That is, we can only hope to visualize these structurally distinct intermediates, each at a specific discrete location on the reaction coordinate. We cannot visualize all structures, continuously distributed along the reaction coordinate. In contrast, a conventional movie reveals a continuous distribution of structures. As an actor walks across the stage, we see his legs moving continuously rather than jerkily; there is a smooth distribution of leg positions, each corresponding to roughly the same energy. Even if we could perform X-ray structural investigations of a single isolated molecule, rather than of the aggregate of 10^{12} molecules in a typical crystal, we would still see structurally distinct intermediates, interconverting in a jerky motion. (As an aside, we note that single-molecule spectroscopies also seem to see only these intermediates. That is, the ergodic theorem holds: the time average over the trajectory of a single molecule corresponds to the instantaneous space average over an ensemble of molecules).

A sixth and critical final component of a time-resolved experiment is therefore to extract the set of time-independent structures, each corresponding to a significantly populated intermediate, from the time-dependent X-ray data. Data that are highly redundant in both time and reciprocal space, and hence adequate to pursue this problem (Moffat, 1989), are just beginning to be acquired.

3. Approaches that seek to trap intermediates

Strategies for time-resolved crystallography depend on the lifetime of the intermediates whose structures are to be determined (Mozzarelli & Rossi, 1996). The traditional approach, denoted 'chemical trapping' (Moffat & Henderson, 1995; Stoddard, 1996), is to prepare indefinitely stable chemical variants of each intermediate, which can then be studied by conventional static crystallographic techniques. This strategy was the first to be adopted (*e.g.* Blake *et al.*, 1967; Henderson, 1970; Blow *et al.*, 1992) and continues to prove powerful, as in a recent study of the mechanism of action of dihydrofolate reductase, DHFR. The reaction catalyzed by DHFR passes through five kinetically and structurally distinct intermediates. Sawaya & Kraut (1997) prepared a set of six isomorphous stable structures designed to simulate each intermediate and a transition state. They could then piece together a six-frame 'movie' of the enzymatic mechanism. A second example is provided by halo-alkane dehalogenase, whose activity (in common with that of most other enzymes) depends markedly on pH and temperature. The structure of substrate-bound enzyme could be obtained at pH 5.0 and 4°C; that of an intermediate subsequently shown to be covalently bound at pH 5.0 and room temperature; and that of the nonconvalently bound chloride product at pH 6.2 and room temperature (Verschueren *et al.*, 1993).

The traditional chemical trapping approach may require quite extensive structural perturbations in order to prolong the lifetime of the intermediate analogs into the tens of hours range. With the arrival of intense synchrotron X-ray sources in which a typical monochromatic oscillation exposure time is tens of seconds or less, less-extensive structural perturbations of the reactants, or of the protein itself *via* site-specific mutagenesis, are necessary. Judicious choice of mutants *via* careful enzymological studies in solution and in the crystal can identify those mutations that enhance the rate of formation or reduce the rate of breakdown of particular intermediates, and hence both stabilize the intermediates and increase their peak populations. A comprehensive study of the enzyme isocitrate dehydrogenase (IDH) by Stoddard, Koshland and co-workers combines site-specific mutagenesis, time-resolved Laue crystallography, molecular-dynamics studies and optical spectroscopy to identify and structurally characterize most intermediates (Stoddard & Koshland, 1993; Bolduc *et al.*, 1995; Stoddard *et al.*, 1996; Mesecar *et al.*, 1997). However, no enzyme variants or other experimental solution conditions could be found that stabilized the enzyme–product complex. Irreversible photoactivation of caged isocitrate substrate, or of either affinity caged or catalytically caged NADP cofactor, led to a single enzymatic turnover in the crystal in which the half-life of the desired enzyme–α-keto-glutarate–Mg^{2+}–NADP product complex was around 10 ms. The structure of this relatively fleeting complex could then be determined by millisecond time-resolved Laue crystallography techniques (Stoddard *et al.*, 1998). This illustrates a general strategy: if you can't slow down the reaction rates, speed up the crystallography! In this example, photoactivation involves irreversible rupture of a covalent bond linking the cage to the substrate or cofactor and, hence, each Laue diffraction image had to be obtained on a separate crystal. The complete data set contained only six such images over an angular range of 30°, yet in the favorable tetragonal space group that these crystals occupy, $P4_32_12$, data sets of around 90% completeness, with very high redundancy and accuracy, could be obtained to 2.1 Å resolution. Stoddard *et al.* (1998) also provide a thoughtful description of the pitfalls of such experiments and of how they successfully avoided them.

As an alternative to chemical trapping, 'physical trapping' (Moffat & Henderson, 1995; Stoddard, 1996) is increasingly employed, in which intermediates are stabilized at reduced temperature by cryogenic techniques (Garman & Schneider, 1997). There are two variants: the more commonly applied 'freeze-trapping', in which the crystal is first frozen and then a structural reaction is initiated, for example by illuminating it with a light pulse; and 'trap-freezing', in which a structural

reaction is initiated at a temperature where the buffer in the crystal is still liquid, the reaction is allowed to proceed for a carefully controlled time to populate a desired intermediate and the crystal is then rapidly frozen. Freeze trapping and trap freezing are distinctly different, owing to the substantial dependence of the nature and extent of structural change – conformational relaxation – on temperature. Indeed, crystallographic cell dimensions and structure itself often depend on temperature (Parak *et al.*, 1987; Frauenfelder *et al.*, 1987; Tilton *et al.*, 1992; Rasmussen *et al.*, 1992; Kurinov & Harrison, 1995). Ideally, the former approach will retain the identical reaction mechanism to that at physiological temperatures but all reaction rates will be greatly diminished and normally short-lived intermediates will become experimentally accessible. For example, the diffusion of the CO ligand that has photodissociated from the heme of myoglobin is slowed by a factor as large as 10^{11} on cooling from room temperature to 40 K (Teng *et al.*, 1997). Diffusion of the CO ligand, or its hopping between two structurally distinct sites in the ligand pocket, occurs on the time scale of hours rather than nanoseconds. If, however, the temperature dependence of all rate constants for the formation and breakdown of each intermediate is identical, the peak population of that intermediate is independent of temperature; only the time after reaction initiation at which the peak population is attained is dependent on temperature (Schlichting & Goody, 1997).

It can be very difficult in the 'freeze-trapping' approach to demonstrate that the intermediates that may be readily visualized experimentally at cryogenic temperatures are structurally identical to those at physiological temperatures, merely longer-lived. That is, cryogenic crystallography may be more precise, in the sense that it affords longer, stronger X-ray exposures from which better structure amplitudes can be derived, perhaps to higher resolution, than at physiological temperatures; but it may be less accurate, in the sense that the structures derived from these amplitudes may differ qualitatively and significantly from those at physiological temperatures (Moffat, 1998). One route to such a demonstration is to compare the nature of spectrally distinct intermediates at cryogenic and physiological temperatures by ultrafast and static spectroscopic techniques. When this strategy is applied to photoactive yellow protein, distinct spectral differences are seen (Imamoto *et al.*, 1996; Schlichting & Berendzen, 1997) that may correlate with structural differences (Perman *et al.*, 1998; Genick *et al.*, 1998; Garber, 1998).

The 'trap-freezing' approach does not suffer from these limitations, since the structural reaction is allowed to proceed initially under near-physiological temperatures in which conformational relaxation can occur. However, the rate at which crystals of dimensions suitable for high-resolution crystallography can be frozen is limited (Teng & Moffat, 1998), ultimately by

the rate of heat transfer from the interior of the crystal to its surface and thence to the cryogen. This is exactly the same physical problem encountered in a minimization of the temperature rise arising from reaction initiation, discussed above. Crystals of dimensions around 100 μm seem to require tens of milliseconds to be completely frozen and this imposes a lower limit on the lifetime of the intermediates that can be trapped and studied crystallographically by this approach. Samples for electron microscopy, on the other hand, are much smaller, a few micrometres in thickness, and can be frozen very much more rapidly, in microseconds (Moffat & Henderson, 1995). That is, the time resolution of this approach is limited by the freezing time and not by the X-ray experiment itself.

The most challenging form of time-resolved crystallography is the 'no-trapping' approach. No chemical or physical manipulation of the crystal is applied and potential structural and mechanistic artefacts associated
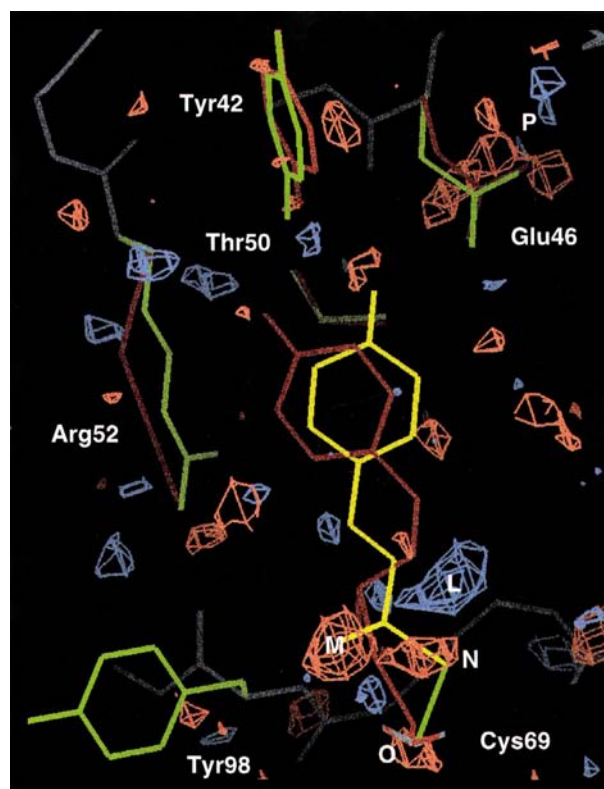

Fig. 1. $|F|_{1\,ns} - |F|_{dark}$ difference-Fourier map of the chromophore region of PYP, where the structure amplitudes obtained 1 ns after illumination of the crystal ($|F|_{1\,ns}$) are compared with those in the dark, prior to illumination ($|F|_{dark}$). The difference map is superimposed on the dark structure (Borgstahl *et al.*, 1995) with important side chains in green and the chromophore in yellow. The model for the 1 ns structure is shown in maroon. Features in the difference map that exceed $\pm 2.6\sigma$ are shown as basket-weave contours; blue denotes positive and red denotes negative. Reprinted with permission from Perman *et al.* (1998). Copyright (1998) American Association for the Advancement of Science.

with these approaches are therefore avoided. However, fast crystallographic techniques must be devised to ensure that X-ray exposure times are less than the life-times of the intermediates. If structural intermediates whose lifetimes are shorter than a few microseconds are to be examined, then the pulsed nature of synchrotron sources can be exploited to yield Laue X-ray exposures as short as 150 ps (Szebenyi *et al.*, 1988, 1992; Bourgeois *et al.*, 1996). Structural changes on this very short time scale are most readily initiated by illumination of the
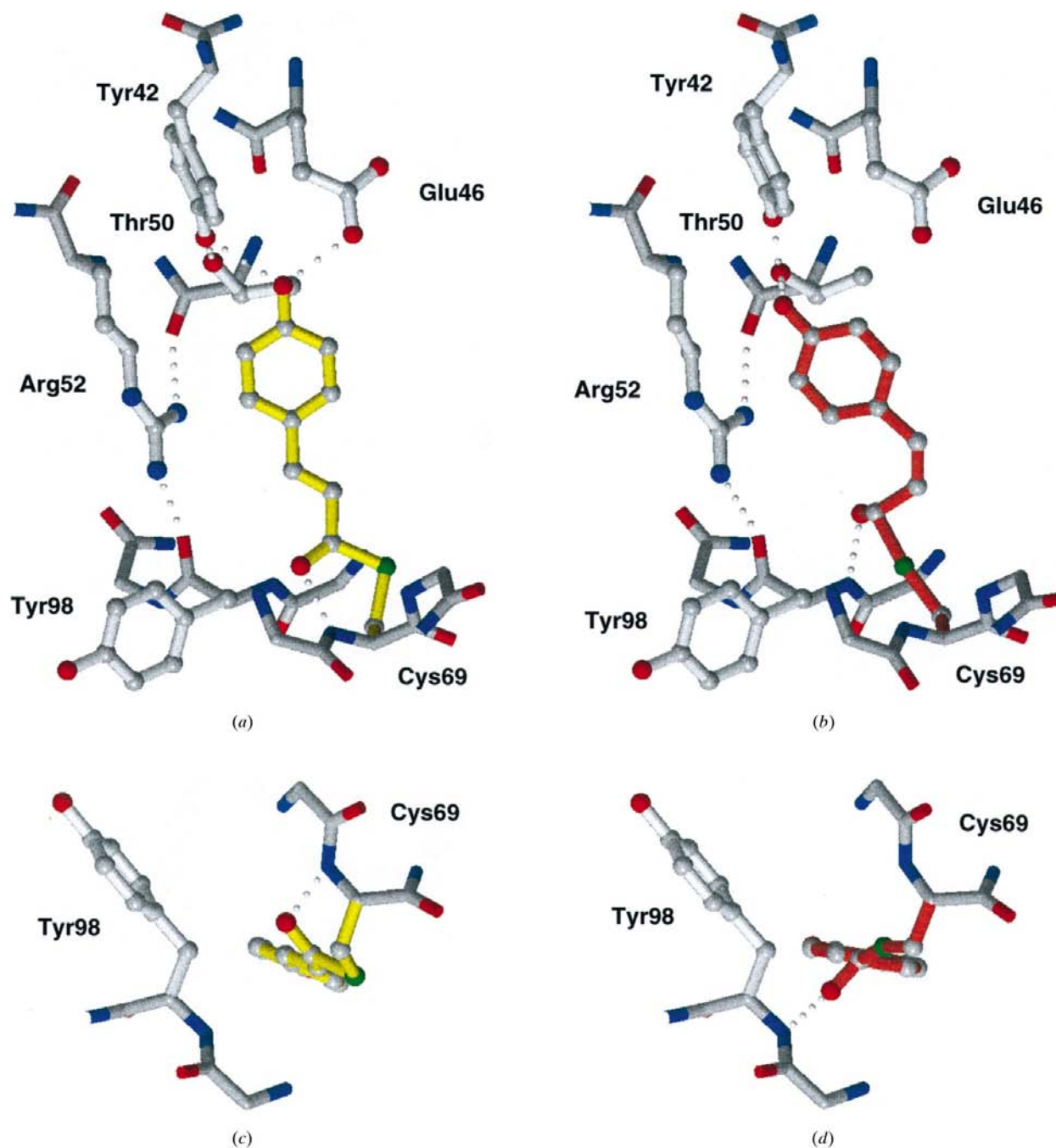

Fig. 2. Comparison of the chromophore region of the dark state and 1 ns structures, indentified with the pG and pR spectroscopic states, respectively (Perman *et al.*, 1998). The pG state structure is shown in two views in (*a*) and (*c*), and the pR state structure in the same two views in (*b*) and (*d*). Key hydrogen bonds are shown dotted. Reprinted with permission from Perman *et al.* (1998). Copyright (1998) American Association for the Advancement of Science.

crystal with a brief laser pulse, for those cases where a fully reversible or irreversible reaction can be initiated by light (Schlichting & Goody, 1997). As might be expected, the no-trapping ultra-fast crystallography approach has its own experimental challenges and potential artifacts. These largely center on the effective acquisition of Laue diffraction patterns (Moffat *et al.*, 1984; Helliwell, 1984; Hajdu, Acharya *et al.*, 1987; Hajdu, Machin *et al.*, 1987; Moffat, 1997), on the accurate extraction of complete sets of structure amplitudes from weak crowded mosaic Laue diffraction patterns (Cruickshank *et al.*, 1987; J. R. Helliwell *et al.*, 1989; Ren & Moffat, 1995*a,b*; Ren *et al.*, 1996; Clifton *et al.*, 1997; Bourgeois *et al.*, 1997, 1998; Yang *et al.*, 1998) that can reveal small structural changes (Lindahl *et al.*, 1992; M. Helliwell *et al.*, 1989); on the development of fast X-ray shutter trains to isolate individual X-ray pulses; on the timing logic to synchronize laser and X-ray exposures and to arm the X-ray detector (Bourgeois *et al.*, 1996); and on minimizing thermal and other sources of crystal disorder that arise on repeatedly stimulating a crystal by an intense laser pulse, while seeking to stimulate as large a fraction of molecules as possible in a spatially uniform nondestructive manner. The sources of artefacts have now largely been identified and the experimental problems overcome. The time resolution of macro-molecular crystallography has been extended from seconds or longer (see *e.g.* Hajdu, Acharya *et al.*, 1987; Schlichting *et al.*, 1990; Gouet *et al.*, 1996), initially to 10 ms in applications to PYP (Ng *et al.*, 1995; Genick *et al.*, 1997) and IDH (Stoddard *et al.*, 1998) and then to a few nanoseconds on both myoglobin (Srajer *et al.*, 1996) and PYP (Perman *et al.*, 1998) in experiments at the European Synchrotron Radiation Facility (ESRF). That is, the time resolution of high-resolution macro-molecular crystallography has been extended by roughly nine orders of magnitude. The fourth dimension, time, has been added to the three spatial dimensions of crystallography; it can no longer be regarded as purely a static technique.

4. Myoglobin

Photolysis of the carbon monoxide complex of myoglobin, MbCO, generates a photoproduct denoted Mb*, in which the ligand CO progressively moves away from the heme, the heme relaxes to the five-coordinate deoxyMb state and the globin also relaxes towards deoxyMb. These processes compete with rebinding of CO and re-relaxation of the heme and globin towards the reactant MbCO state. The entire process is fully reversible in the crystal and is complete in roughly 200 μs (Srajer *et al.*, 1996). It has been examined over many years by a wide range of spectroscopic techniques and by molecular dynamics and other computational approaches to which the crystallographic results can be related [see citations in Srajer *et al.* (1996) and the discussion in Eaton *et al.* (1996)]. That is, the results put a structural foundation under the spectroscopic infer-ences and under the computations, which were of necessity previously based on static structures only. They visualize directly the loss of the CO and its rebinding, with a concomitant motion of the Fe atom relative to the heme plane; they reveal a putative 'docking site' in the ligand pocket for the outgoing CO that lies neatly on the overall trajectory for the CO seen more clearly in parallel cryogenic experiments (Teng *et al.*, 1994; Schlichting *et al.*, 1994; Hartmann *et al.*, 1996; Teng *et al.*, 1997), and which in turn agrees with computational approaches (Vitkup *et al.*, 1997); and they appear to reveal the beginnings of relaxation of the globin, though the crystallographic signal-to-noise ratio was not sufficient for unambiguous interpretation in the earliest data. Numerous further data sets that span the complete time course of this reaction will enable the true nature of the time course to be assessed (single, multiple or stretched exponential) and, with luck, reveal more accurate details of structural relaxation in the globin. However, nature may have conspired against us: re-binding of CO occurs in direct competition with globin and CO relaxation and the fully relaxed deoxyMb structure may not be attainable before significant rebinding occurs in wild-type myoglobin. This property may differ in the series of variants of myoglobin prepared by Olson & Phillips (1996) with altered ligand-binding properties and possibly also altered rates of globin relaxation.

5. Photoactive yellow protein

PYP is a particularly simple, small (14 kDa), water-soluble, bacterial, blue-light photoreceptor that under-goes an efficient fully reversible photocycle on absorbing a photon (Meyer *et al.*, 1987). It contains a 4-hydroxycinnamic acid chromophore covalently linked to the sole cysteine in PYP through a thioester bond (Hoff, Diix *et al.*, 1994; Baca *et al.*, 1994). This photocycle contains a ground state denoted pG and at least two spectrally distinct intermediates denoted pR and pB that presumably differ in tertiary structure (Meyer *et al.*, 1987, 1989; Hoff, Van Stokkum *et al.*, 1994). PYP therefore converts light energy into chemical energy – one or more structural signals – and ultimately into a biological response, altered swimming behavior of the bacterium *Ectothiorhodospira halophila* from which PYP was originally isolated (Meyer, 1985). It is there-fore of considerable biological interest and, since the intermediates appear to span the time range from sub-picoseconds to seconds, ideally suited for biophysical studies. The overall scientific question is: what is the structural basis for signal transduction in the PYP system? The putative downstream receptor(s) in this signal transduction pathway are unknown and studies so far have concentrated on PYP itself.

The correct structure of PYP was finally obtained at 1.4 Å resolution (Borgstahl *et al.*, 1995) and recently at 0.85 Å resolution at cryogenic temperature (Genick *et al.*, 1998). We studied initially the establishment of and decay from a saturated photostationary state of PYP from the BN9626 strain of *E. halophila* (Ng *et al.*, 1995; Ren *et al.*, 1996; Genick *et al.*, 1997). The 4-hydroxycinnamic acid chromophore of PYP is *trans*, unprotonated on its phenolate oxygen and completely inaccessible to solvent in the ground pG state (Borgstahl *et al.*, 1995), but is *cis*, ejected from the chromophore pocket, accessible to solvent and protonated in the photostationary state, identified with the pB intermediate. Time-resolved crystallographic data were obtained with 10 ms time resolution at the National Synchrotron Light Source, Brookhaven, during the reversion of pB back to pG in the last phase of the photocycle [Genick *et al.* (1997), and unpublished results of the Moffat and Getzoff groups]. The structural changes in pB, the presumptive signaling state, were largely confined to the chromophore, its pocket and the adjacent protein surface, and were clearly of a sufficient magnitude to alter the affinity of PYP for other macromolecules in passing from the pG to the pB state and thus to generate a structural signal. However, these studies did not reveal how the pB state was generated, or the stage in the photocycle at which the *trans* to *cis* isomerization and the changes in protein structure occurred. Identification of these features required a nanosecond time-resolved crystallographic study, conducted at the ESRF. The structure at the shortest time delay after initiation of the photocycle by a brief laser pulse, 1 ns after absorption of a photon and characteristic of the pR state, clearly showed that isomerization had occurred, that the carbonyl oxygen of the chromophore tail was repositioned, that a very specific restructuring of hydrogen bonds which position the chromophore in its pocket had occurred, but that the chromophore remained in its pocket (Perman *et al.*, 1998). Tertiary structural changes in the protein were largely confined to those regions of the pG state that possess unusual secondary structure. It is as though the pG state is cocked, ready to absorb a photon and to undergo specific structural changes with high quantum efficiency without dissipating significant energy in fluorescence or in heat. In this regard, PYP is diametrically opposite to green fluorescent protein, GFP, whose intrinsic protein-derived chromophore bears an interesting resemblance to that of PYP (Figs. 1 and 2; Perman *et al.*, 1998). For GFP, the energy of the absorbed photon appears with high quantum efficiency in fluorescence. As with myoglobin, a complete understanding of the photocycle mechanism of PYP will require that the temporal progression of structural features in both the chromophore and its environment be examined – the molecular movie. One circuit of the PYP photocycle requires about 1 s, yet spectral changes are evident on the picosecond time scale. Thus, time-resolved crystallography on PYP will have to probe 12 decades of time. With such data in hand, the major remaining crystallographic and computational challenge in time-resolved crystallography can be attacked: from time-dependent X-ray data that are highly redundant both in time and in reciprocal space, to identify and extract the time-independent structures, each corresponding to a distinct intermediate. Whether this extraction can be achieved more efficiently in real space or reciprocal space, or indeed at all, remains to be seen; it is a long-sought goal.

Both myoglobin and PYP exhibit spectral changes on time scales shorter than those presently probed by nanosecond laser pulses and, indeed, shorter than the 100 ps duration of the X-ray pulses from today's synchrotron sources. Probing even shorter-lived structural species will therefore require either time-slicing of synchrotron pulses or generation of novel X-ray sources that afford pulses in the picosecond range or below. Advances are being made in this direction *via* several approaches (see *e.g.* Tomov *et al.*, 1995; Schoenbein *et al.*, 1996; Schneider, 1997). For such approaches to become useful for time-resolved crystallography, the brilliance of the sources will have to be substantially increased, in a useful wavelength range, to match the diffraction characteristics of the crystals. Nevertheless, those novel approaches probably offer the best promise for ultrafast diffraction experiments, always supposing that the crystals will withstand the exceptional peak powers required, without dielectric breakdown or other prompt irreversible radiation damage.

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