the single and multiple data are merged and reduced to structure amplitudes. Ren *et al.* (1999) have emphasized that, in contrast to the simplified description presented in Section 8.2.2, the effective wavelength range $\lambda_{max} - \lambda_{min}$ depends on resolution, and each Laue spot is stimulated by a range of wavelengths which can be quite large at low resolution. Although typical Laue software packages such as the *Daresbury Laue Software Suite* (Helliwell *et al.*, 1989; Campbell, 1995), *LEAP* (Wakatsuki, 1993) and *LaueView* (Ren & Moffat, 1995*a*,*b*) are largely automated, a surprising degree of manual intervention may still be required in the first (indexing) stage, and in later stages where the order of parameter refinement and various rejection criteria may be adjusted by the user. The overall result is that carefully conducted Laue experiments yield structure amplitudes that equal those from monochromatic data in quality (Ren *et al.*, 1999).

8.2.4. The time-resolved experiment

The principles and applications of time-resolved macromolecular crystallography have been widely reviewed (Moffat, 1989; Cruickshank *et al.*, 1992; Hajdu & Johnson, 1993; Helliwell & Rentzepis, 1997; Moffat, 1998; Ren *et al.*, 1999). This article therefore concentrates on the crystallographic aspects.

The essence of a perfect time-resolved crystallographic experiment is that a structural reaction is initiated in all the molecules in the crystal, rapidly, uniformly and in a non-damaging manner. The molecules, far from thermodynamic equilibrium immediately after the completion of the initiation process, relax through a series of structural transitions back to equilibrium. The course of the structural transitions is monitored through the time-dependence of the X-ray scattering. The structure amplitudes (and indeed the phases) associated with each Bragg peak *hkl* become time-dependent and may be denoted $|F(hkl,t)|$. The Fourier transform of the structure factors yields the time-dependent space-average structure of the molecules in the crystal. If all molecules behave independently of one another in the crystal, as they do in dilute solution, then the overall time dependence arises from the time dependence of the fractional populations of each time-independent structural state. That is, the crystal exhibits time-dependent substitutional disorder. (Lest this seem an unfamiliar concept, recall that a multi-site, partially occupied, heavy-atom derivative also exhibits substitutional disorder: the contents of each unit cell differ slightly, depending on whether a particular heavy-atom site is occupied in that unit cell or not. Such disorder fortunately does not invalidate the use of that derivative. The analysis proceeds as though a particular site were, say, 70% occupied in every unit cell of the crystal, although in reality that site is 100% occupied in 70% of the unit cells. In this example, the substitutional disorder is time-independent.) The crystal is thus imperfect: substitutional disorder breaks the translational symmetry. It follows that there must also be time-dependent non-Bragg scattering; but all studies to date have focused on the Bragg scattering.

The above describes a perfect experiment but, as might be expected, reality is different. Initiation techniques such as absorption of light from a laser pulse (Schlichting & Goody, 1997) unavoidably deposit energy in the crystal and give rise to a temperature jump and transient, reversible crystal disorder, evident as spot streaking. The magnitude of this temperature jump is proportional to the number of photons absorbed, which in turn is related to the concentration of photoactive species, the quantum yield for photoactivation and the fraction of molecules stimulated (Moffat, 1995, 1998). The necessity for limiting the magnitude of this temperature jump to retain crystallinity means that it is difficult to initiate the reaction in all molecules in the crystal. For example, photodissociation of carbon monoxide from carbonmonoxymyoglobin crystals was achieved in roughly 40% of the molecules (Šrajer *et al.*, 1996), and entry into the photocycle of photoactive yellow protein in roughly 20% of the molecules (Perman *et al.*, 1998). The magnitude of the time-dependent change in structure-factor amplitudes, given by

$$\Delta F(hkl,t) = |F(hkl,t)| - |F(hkl,0)|,$$

is proportional to the fraction of molecules photoactivated and is therefore substantially diminished.

The main crystallographic challenge thus becomes the accurate determination of small values of $\Delta F(hkl,t)$ in the face of both random errors (arising from, for example, the small numbers of diffracted photons into the reflection *hkl* from a brief X-ray pulse) and systematic errors (arising from, for example, inaccurate determination of the Laue wavelength normalization curve, crystal-to-crystal scaling errors, inadequately corrected absorption effects, or time-dependent spot profiles). Precision is enhanced by acquiring highly redundant Laue data (mean redundancies typically between 5 and 15), which also afford an excellent measure of the variance of the structure amplitudes, and accuracy is enhanced by interleaving measurements of $|F(hkl,t)|$ with those of $|F(hkl,0)|$ on the same crystal, at nearly the same time. Indeed, 'two-spot' Laue patterns may be acquired by recording both the $|F(hkl,t)|$ and $|F(hkl,0)|$ diffraction patterns, slightly displaced with respect to each other, on the same detector [image plate or charge-coupled device (CCD)] prior to readout and quantification (Ren *et al.*, 1996). However, this doubles the background and halves the signal-to-noise ratio.

The values of $\Delta F(hkl,t)$ span a four-dimensional space. What is the best way to scan this four-dimensional space, having regard for the need to minimize errors? Interleaving measurements of $|F(hkl,t)|$ and $|F(hkl,0)|$ has been achieved by fixing the delay time t between reaction initiation (the pump, laser pulse) and X-ray data acquisition (the probe, X-ray pulse), and surveying all values of *hkl* through progressive reorientation of the crystal between Laue images until all the unique volume of reciprocal space is surveyed with adequate redundancy and completeness (Šrajer *et al.*, 1996; Perman *et al.*, 1998; Ren *et al.*, 1999). The value of t is then altered and data acquisition repeated for all suitable values of t. That is, t is the slow variable. The difficulty with this approach is that a single crystal may yield only one or two data sets, corresponding to one or two values of t, before radiation damage (predominantly laser-induced rather than X-ray-induced) compels replacement of the crystal. The entire reaction time course over all values of t must therefore be pieced together from measurements on many crystals, a process which is prone to inter-crystal scaling errors. A second experimental approach to scanning this four-dimensional data space is therefore to fix the crystal orientation, obtain values of $|F(hkl,t)|$ and $|F(hkl,0)|$ for all suitable values of t, reorient the crystal, recollect these same values of t, then replace the crystal and repeat until all the unique volume of reciprocal space is surveyed. That is, *hkl* are the slow variables (B. Perman, S. Anderson & Z. Ren, unpublished results). This approach yields a more accurate time course, but (for a single crystal) from a subset of reflections only. In practice, of the order of 100 time points t may be collected.

The first approach permits Fourier or difference Fourier maps to be calculated using data from a single crystal at one or a small number of time delays t. The second approach requires data from many crystals to be acquired before such maps can be calculated. This complicates the issue of which is the better approach. Preliminary results (V. Šrajer & B. Perman, unpublished results) suggest that genuine features may be reliably distinguished in real space by examination of Fourier and difference Fourier maps, but genuine trends in reciprocal space are much harder to discern.

Table 8.2.5.1. *Time-resolved Laue diffraction experiments*

This table is adapted from Table 2 of Ren *et al.* (1999), in which citations of the original experiments are provided.

Protein	Time resolution	Experiment
Hen lysozyme	64 ms	Temperature jump test
Glycogen phosphorylase	1 s	Bound maltoheptose
Hen lysozyme	1 s	Radiation damage test
Glycogen phosphorylase	100 ms	Use of caged phosphate
Ras oncogene product	1 s	GTP complex
γ-Chymotrypsin	5 s	Photolysis of cinnamate/pyrone
Trypsin	800 ms	Ordered hydrolytic water
Cytochrome *c* peroxidase	1 s	Redox active compound I
Hen lysozyme	10 ms	Temperature jump
Isocitrate dehydrogenase	50 ms	ES complex and intermediate
Isocitrate dehydrogenase	10 ms	Product complex
Photoactive yellow protein	10 ms	*p*B-like intermediate
Photoactive yellow protein	10 ns	*p*R-like intermediate
CO-myoglobin	10 ns	Photolyzed CO species at 290 K
CO-myoglobin	8 ms	Photolyzed CO species at 20–40 K
Hydroxymethylbilane synthase	1.5 ms	Mutant enzyme–cofactor complex

How many features can be distinguished as genuine in real space, in, for example, a difference Fourier map? We presently employ three criteria. First, the feature must be 'significant' in crystallographic terms. That is, its peak height must exceed (say) 4σ to 5σ, where σ is the r.m.s. value of the difference electron density $\Delta\rho$ across the asymmetric unit, in a difference Fourier map. Second, the feature must be chemically plausible, *e.g.* located on or near critical groups in the active site. Third, the feature must persist over several time points. No genuine feature is likely to vary faster than exponentially in time (though slower variation is possible), but noise features tend to come and go, varying rapidly with time. The third criterion, which in effect is applying a low-pass temporal filter to the data, or 'time-smoothing', is only applicable if several time points are available per decade of time t. It is to ensure that this powerful criterion can be applied in an unbiased manner that the time points t at which data are acquired are uniformly and closely spaced in log t.

Suppose that complete and accurate values of $|F(hkl, t)|$ are available to high resolution and at numerous values of t. How can these time-dependent data be further analysed to yield information on the reaction mechanism and the time-dependent structures of intermediates? Each candidate chemical-kinetic mechanism implies a different time-dependent mixture of structural states at all times t. For each mechanism, a set of trial time-dependent intermediate structures can be calculated from the time-dependent data (Perman, 1999). One then asks: Is each trial intermediate structure an authentic, single, stereochemically plausible, refinable protein structure? If so, the mechanism is supported, but if not, the mechanism is rejected. This process, of seeking to extract time-independent structures from time-dependent data, is closely related to the better-understood process of extracting time-independent difference spectra from time-dependent optical absorption data *via*, for example, singular value decomposition or principal component analysis. The latter, optical analysis, proceeds in two dimensions, $\mathrm{OD}(\lambda, t)$; the former, crystallographic analysis, must proceed in four dimensions, either $\rho(xyz, t)$ or $|F(hkl, t)|$.

It will be appreciated that the acquisition of fast, time-resolved data is greatly hindered by the lack of a time-slicing area detector. This lack is even more evident when the structural reaction is irreversible as, for example, in the photoactivation of caged GTP to GTP (Schlichting *et al.*, 1990). In such cases, the reactants must be replenished prior to each reaction initiation, which makes the acquisition of time-resolved data particularly tedious. The present generation of CCD detectors have an inter-frame time delay in the millisecond (or just sub-millisecond) time range. Pixel array detectors under development may permit the acquisition of sequential images with a time delay in the microsecond range. The desirable nanosecond or even picosecond time range seems inaccessible for area detectors (but not for point detectors such as streak cameras). A new approach may be needed, such as the use of chirped hard X-ray pulses which, in combination with Laue diffraction, map X-ray energy into both reciprocal space (*hkl*) and time (K. Moffat, in preparation).

8.2.5. Conclusions

Only a small number of biochemical systems have been subjected to time-resolved crystallographic analysis (Table 8.2.5.1; Ren *et al.*, 1999). The experiments are technically demanding, require careful planning in the execution, in data analysis and in data interpretation, and strategies for the evaluation of mechanism are still being developed. However, road maps exist for several successful classes of experiments (see *e.g.* Stoddard *et al.*, 1998; Moffat, 1998; Ren *et al.*, 1999) and new biological systems to which such analyses may be readily applied are being developed. In a world of structural genomics where structures themselves are ten-a-penny, a structure-based understanding of mechanism at the chemical level is still rare. The contributions of crystallography to functional – not merely structural – genomics may be large indeed.

Acknowledgements

This work was supported by the NIH. I thank Zhong Ren for comments on the manuscript.