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 That is, hkl are the slow variables (B. Perman, S. Anderson & Z. Ren, unpublished results).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 Preliminary results (V. Šrajer & B. Perman, unpublished results) suggest that genuine features may be reliably distinguished in real space by examination of Fourier and difference Fourier maps, but genuine trends in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.

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, OD(λ, 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).

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

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.

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