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Laue Crystallography for Studying Rapid Reactions

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Precise quantitation of static and time-resolved Laue diffraction patterns is undeniably more complex than for monochromatic patterns. Recent advances in integration and scaling algorithms demonstrate that, with suitable care in the conduct of the Laue experiment itself, Laue data sets can be obtained which rival the best monochromatic data sets in accuracy and completeness. These algorithms deal in an integrated fashion with the several main problems of Laue diffraction patterns: the elongated spots which arise from mosaic crystals, the spatial overlaps which occur in crowded diffraction patterns, the energy overlaps which arise from the mapping of a central line in reciprocal space onto a single spot in detector space, and wavelength normalization.

Keywords: Laue diffraction; integrated intensities; spatial overlaps; energy overlaps; wavelength normalization.

Introduction

Since the Laue method was first applied to crystallographic studies of macromolecules (Moffat, Szebenyi & Bilderback, 1984), it has been realized that the Laue method is likely to be the technique of choice in rapid crystallographic kinetic studies and the analysis of time-dependent structural perturbations, due to its twin advantages of the briefest exposure times and the use of a stationary crystal. The principles and recent applications to time-resolved crystallography of both the Laue method and monochromatic techniques have been extensively reviewed by Cruickshank, Helliwell & Johnson (1992), Pai (1992) and Hajdu & Andersson (1993), and we do not in this brief review consider them further. We focus here on a particular problem: the Laue method is undeniably more complex than well established monochromatic methods, and there is disagreement on the extent to which these complexities affect data quality and completeness. Even if excellent time-resolved Laue diffraction patterns can be obtained, the method fails unless excellent time-dependent structure amplitudes can be extracted from them. Helliwell (1992) asserts that the ability of the Laue method to yield accurate integrated intensities and hence good structural data has been established. However, Hajdu & Andersson (1993; see also Andersson *et al.*, 1992) note that the Laue technique has certain limitations which if downplayed or ignored, greatly compromise data quality. The Laue technique is considerably more sensitive than monochromatic techniques to crystal disorder. Certain forms of crystal disorder lead to radially elongated, streaky Laue spots which are more difficult to integrate accurately and which tend to overlap, particularly for crystals with large unit cells (Shrive, Clifton, Hajdu & Greenhough, 1990). More complicated data-reduction strategies must be implemented to account for the X-ray wavelength dependence of the many factors involved in the reduction of raw Laue intensities to structure amplitudes (Helliwell, 1992). Energy overlaps, in which harmonics of a fundamental reflection (*hkl*) are exactly superimposed, are particularly evident for low-resolution reflections (Cruickshank, Helliwell & Moffat, 1987), and the Ewald spheres corresponding to $1/\lambda_{min}$ and $1/\lambda_{max}$ closely approach each other at low resolution. A Laue data set may therefore be quite incomplete at low resolution (the 'low-resolution hole'), which seriously perturbs resulting Fourier and difference Fourier maps (Duke *et al.*, 1992). These limitations led Hajdu & Andersson (1993) to state 'As it stands the Laue method is not the method of choice for time-resolved structural studies.' The key phrase in this quotation is 'as it stands'; the problems with the Laue method that Hajdu & Andersson (1993) identify are real, and the question is whether they can be overcome by suitable improvements in the Laue method over the way it is presently practiced. We believe that the answer is yes. Table 1 identifies each of these problems and their consequence, and suggests both experimental and computational solutions.

Problems such as crystal mosaicity may be exacerbated in time-resolved experiments and, in extreme cases, the crystal may become so disordered that the diffraction pattern can no longer be recorded, let alone accurately quantitated. A final problem is common to the analysis of all time-resolved crystallographic experiments: the diffraction pattern represents an average over all molecules in the crystal and over the duration of the X-ray exposure. That is, at a minimum the crystal exhibits time-dependent substitutional disorder, since it contains a mixture of structural states whose populations vary with time. The final goal of the data analysis is to identify each of these time-independent structural states. Although one strategy for proceeding with this identification in reciprocal space has been advanced (Moffat, 1989), this strategy has not yet been

Table 1
Problems in the Laue method and their solution.

Problem	Consequence	Experimental solution	Computational solution	Notes
Wavelength normalization necessary to extract structure amplitudes	More complicated, error-prone data processing	Avoid wavelength normalization through use of $\Delta F/F$ values	Improved algorithms for data analysis, *e.g.* use of Chebyshev polynomials to model spectra	Experimental solution does not yield complete data sets; Helliwell *et al.* (1989), Ren & Moffat (1994*a*,*b*)
Energy overlaps	Certain low-order and other reflections not measurable; data systematically incomplete at low resolution	Set $\lambda_{max} \leq 2\lambda_{min}$ to minimize energy overlaps and low-resolution 'hole'	Devise algorithms to deconvolute energy overlaps	Experimental solution replaced by computational solution; Helliwell *et al.* (1989), Campbell & Hao (1993), Ren & Moffat (1994*b*)
High sensitivity to crystal mosaicity	Streaky spots which are not readily integrated	Identify and where possible minimize source of mosaicity: intrinsic (light-induced, diffusion-induced, gradient-induced), X-ray-generated; collect data before disorder appears	Improved algorithms for accurate integration of streaky spots	Shrive *et al.* (1990), Wakatsuki (1993), Ren & Moffat (1994*a*)
Dense patterns with many spots too close together to resolve; numerous spatial overlaps	Incomplete data sets; restriction on unit-cell sizes	Restrict λ range; employ 'toast rack' detector	Improved algorithms for integration of closely adjacent or overlapping spots	Cruickshank *et al.* (1991), Helliwell (1992); experimental solution replaced by computational solution; Ren & Moffat (1994*a*), Shrive *et al.* (1990)
Few angular settings of crystal employed	Incomplete low-resolution data	Revise data-collection scheme		May also affect accuracy of wavelength normalization curve and ability to deconvolute energy overlaps
Radiation sensitivity of the crystal	Loss of high-resolution data; streaky spots	Restrict λ range to minimize crystal heating; cool or freeze crystal		A restricted λ range may require more images to complete data set
Necessity for time-resolved data collection	More elaborate data-collection protocols	Improved time-slicing detectors; use of integrating detectors on streak stage; rapid detector interchange mechanisms	Improved algorithms for analyzing time-dependent data	Ren *et al.* (1994)
Crystal contains time-varying mixture of structural states; substitutional or other disorder	Superposition of structures visualized; possible loss of diffraction quality; enhanced diffuse scattering	Monitor distribution of states independently, *e.g.* by optical analysis; devise conditions such that individual states are sequentially populated to a large extent	Devise algorithms for deconvoluting mixed structures	Principles discussed by Moffat (1989); still to be implemented

attempted since suitable data have been lacking until very recently. Related strategies can be devised in real space (K. Moffat, unpublished results). An alternative experimental approach, which is clearly preferable if it can be arranged to apply to the crystal in question, is to identify time periods after initiation of a structural reaction when a particular structural state predominates. One example is provided by the photocycle of photoactive yellow protein, PYP. Species in the photocycle of PYP interconvert *via* sequential, first-order reactions in which the rate constants progressively diminish, and differ by several orders of magnitude [see Figs. 3 and 4 of Moffat, Chen, Ng, McRee & Getzoff (1992)], with the result that time periods can be identified when all the PYP molecules are in each structural state.

Hajdu & Andersson (1993), in reviewing recent progress, issue a call to arms: 'We suggest that one of the major goals of structural biology should be to achieve four-dimensional structure determination, with time being the fourth dimension, and four-dimensional structures and coordinate files being the norm, in which conventional three-dimensional structures are stills from the movie.' Although it is a real challenge to execute a time-resolved (or four-dimensional) experiment correctly and four-dimensional structures are by no means the norm, it is our thesis that with great care in experimental design, they are attainable. For all but the slowest reactions, for which monochromatic techniques may suffice, accurate and complete Laue structure amplitudes are essential if this goal is to be achieved.

Quantitation of static and time-resolved Laue diffraction patterns

There are two main Laue analysis packages in use, one originating at the Daresbury Laboratory (Helliwell *et al.*, 1989) and one at Cornell (Smith Temple, 1989). A third (Wakatsuki, 1993) is under development at Oxford. We have developed a new integrated approach to Laue analysis (Ren & Moffat, 1994*a*) and encoded it in a software package denoted *LaueView*.

The approach was designed from the outset to tackle both static (one-spot) and time-resolved (two or more spot) Laue data, and to deal in an integrated manner with the complexities noted above of the Laue method: spatial overlaps (Cruickshank, Helliwell & Moffat, 1991), streaky spots from mosaic crystals, and wavelength normalization. A surprisingly straightforward extension (Ren & Moffat, 1994*b*) enables the final complexity, energy overlaps, to be conclusively addressed, and thus removes the 'low-resolution hole' which has compromised earlier analyses. The approach [details of which are in Ren & Moffat (1994*a*,*b*) and Ren, Moffat, Getzoff, Borgstahl & Genick (1994)] involves modeling of the profile of all reflections by a flexible, multi-parameter, analytical profile whose mathematical form is chosen to enable streaky or other odd-shaped spots to be fit. The values of the parameters describing a profile are derived *via* a non-linear least-squares process from local sample reflections and are constrained to vary smoothly and slowly across the image. Rather than fitting a single profile to each single reflection in turn, a linear least-squares process fits simultaneously the integrated intensities of all reflections in a physically contiguous region. This region includes spots which overlap either because they are part of the same Laue pattern, or because they belong to a different time point of another pattern. Thus, in contrast with the approach of Shrive *et al.* (1990), all detector pixels are used in the fitting, including those with contributions from one or more spots. Shrive *et al.* (1990) suggested an approach to deconvolution of spatially overlapping reflections based on identification and rejection of those pixels that form part of the profiles of two or more spots. First, it is critical to the success of this approach to identify correctly if a pixel is involved in more than one reflection. Especially for a noisy diffraction pattern, this is hard to perform accurately, and it must rely heavily on certain rejection criteria. Second, rejection of some pixels reduces the data-to-parameter ratio and increases the uncertainty of the integrated intensity. Our approach models all pixels both in overlapping and non-overlapping areas; they contribute equally to the profile fitting. When spatially overlapping reflections are completely modeled, they are resolved.

Wavelength normalization is modeled by Chebyshev polynomials of adjustable degree, which has proved very successful (Smith Temple, 1989) in modeling both smooth and sharply featured spectra, the former from a bending magnet or wiggler source and the latter from an undulator (Szebenyi *et al.*, 1992). Scaling by this route employs a generalized non-linear least-squares process using the Levenberg–Marquardt algorithm and singular value decomposition. Chebyshev polynomials are chosen because of their minimax property, which means that in practice one can gradually increase the maximum degree to fit sharp features in X-ray spectrum, or reduce it later if, for example, noise appears in the wavelength normalization curve. Altering the maximum degree results in minimum re-adjustment of the previously obtained coefficients. A widely used alternative is the wavelength binning method (Helliwell *et al.*, 1989), but this technique has certain shortcomings. Use of small wavelength bins simultaneously increases the total number of parameters and decreases the data in each bin, which greatly increases the uncertainty of the scale factors for each bin. Use of larger wavelength bins means averaging over a larger wavelength range, which also increases the uncertainty of the scale factor. Therefore, a bin size which is a (generally fixed) compromise between these factors must be used.

With accurate values of scale factors in hand from analysis of single reflections [those stimulated by only a single X-ray wavelength and associated with a single reciprocal lattice point (Cruickshank *et al.*, 1987)], the problem of energy overlaps can be addressed. The raw integrated intensity of a multiple reflection (stimulated by several wavelengths and associated with several reciprocal lattice points along a ray) is clearly given by the sum of the raw integrated intensities associated with each component of the multiple. With sufficient observations at different crystal settings of the raw integrated intensities of the multiple reflection, it proves readily possible (Ren & Moffat, 1994*b*) to fit the values of the structure amplitudes for each component, in a nonlinear least-squares process which involves all observations and all multiplicities simultaneously. Our approach differs in important aspects from that of Campbell & Hao (1993). First, our approach utilizes all observations of multiple reflections, whether containing one component or as many as 30. Other deconvolution approaches (Helliwell *et al.*, 1989; Campbell & Hao, 1993) deal with only low-order multiple reflections containing up to three components. They do not incorporate those reflections in the approaches which are single in some images but multiple in others, nor those multiple reflections which contain more than three components. Consideration of the first is critical to the deconvolution process, and that of the second is helpful. Second, solving for the squared values of structure amplitudes may lead to negative solutions (Campbell & Hao, 1993) from which structure amplitudes cannot be extracted. Such reflections must be eliminated on mathematical rather than physical grounds. We obtain (Ren & Moffat, 1994*a*,*b*; Ren *et al.*, 1994) Laue amplitudes which are both complete (completeness in each resolution shell, including that between $2d_{\min}$ and ∞, exceeding 90%) and accurate, with scaling R factors *versus* reference monochromatic data in the 4–8% range (unweighted) and in the 2–5% range (weighted) for both singles alone and for the components of deconvoluted

multiples. The higher values are found for tiny mosaic crystals of PYP in a time-resolved experiment.

This approach to the analysis of time-resolved Laue data requires that each observation be scaled and subjected to wavelength normalization separately. An alternative approach (Bilderback, Moffat & Szebenyi, 1984; Hajdu *et al.*, 1987) avoids the need for scaling and compares directly the raw integrated intensities before and after applying a perturbation to the crystal, to yield values of $\Delta F/F$, the fractional change in amplitudes associated with the structure perturbation. We have compared these two approaches on the same PYP data set (Ren *et al.*, 1994). Both yield accurate difference coefficients ΔF, but the latter approach cannot resolve the components of multiple reflections and thus yields Laue data sets of somewhat lower completeness.

The time scale of Laue experiments ranges from 100 ps (Szebenyi *et al.*, 1992) to minutes [see, for example, Table 1 of Hajdu & Andersson (1993)]. This spans the range of structural processes from highly localized tertiary structural changes such as aromatic ring flips (ns) to larger-scale domain motion associated with the lifetimes of enzyme-substrate intermediates (ms–s) or intermediates in photocycling. In some biochemical systems, even slower structural processes (s–min) can be readily visualized. A critical parameter in designing time-resolved experiments is the time resolution, which must be shorter than the lifetime of the shortest-lived species that accumulates and whose structure is to be determined. The time resolution may be set by the method of reaction initiation. For example, diffusion of reactants into crystals requires many tens of seconds; but pulsed laser initiation can, if necessary, be achieved on a time scale well under 1 ns. Alternatively, the time resolution may be set by the minimum X-ray exposure necessary to achieve an excellent diffraction pattern. This ranges from the duration of a single X-ray pulse, 50–100 ps, to the elapsed time occupied by a train of pulses. It is clear that, for the slower processes, the well established monochromatic X-ray techniques are ideal; and that for the fastest processes, only Laue techniques will suffice. As rapid-rotation monochromatic techniques are developed, they may be extended to shorter time scales and better time resolution. The time resolution at which monochromatic techniques give way to Laue techniques will vary, as better time-slicing detectors and more intense X-ray sources are introduced. Nevertheless, the fact remains that a significant (and preferably random) sampling of all reflections in the unique volume in reciprocal space must be achieved at all time points, whatever method is employed. It is simply not the case that the speed advantage of the Laue method over monochromatic techniques is lost when more than one Laue exposure needs to be taken to sample the unique volume and complete the data set. The acquisition of further Laue (or monochromatic) exposures is completely without effect on the time resolution, though of course it does increase the total time to complete the experiment.

We believe that, with care in the design of the Laue experiment itself and with the application of these new data-processing strategies, Laue data sets which are both accurate and complete can be obtained. Since their accuracy and completeness are entirely comparable with those of high-quality monochromatic data sets, the choice of whether to pursue a time-resolved experiment by Laue or monochromatic techniques now hinges on suitability for the experiment in question and in particular on its time scale, rather than on the quality of the structure amplitudes which will be obtained.

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