Strategies for macromolecular synchrotron crystallography

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Structure 2000, 8:R105–R110

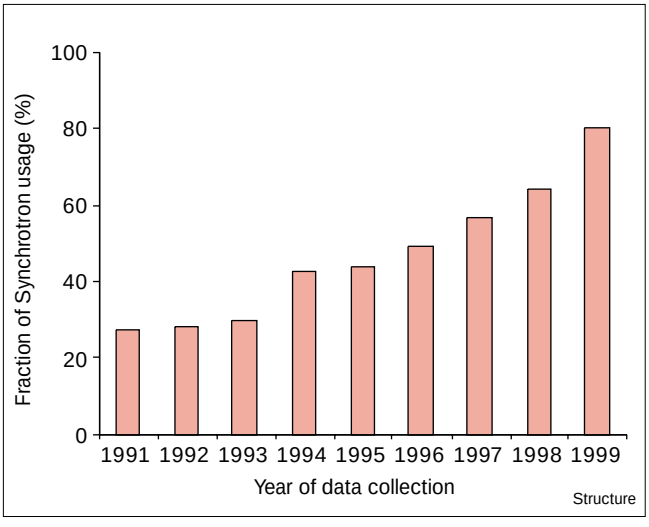
0969-2126/00/$ – see front matter

Introduction

During the past ten years the rate of protein structure determination by X-ray crystallography has risen about tenfold [1]. The use of synchrotron radiation was instrumental for this growth and the fraction of the results obtained this way increased from 28% in 1990 to 80% in 1999 (Figure 1). There are 52 synchrotron stations worldwide used for macromolecular crystallography, and there are more than 30 stations under construction or to be built in the near future. The increase in the number of radiation stations was less of a factor in the growth of the results, however, than the improvements in beam intensity and tunability, detectors (Figure 2), computational capacity, software and data collection protocols.

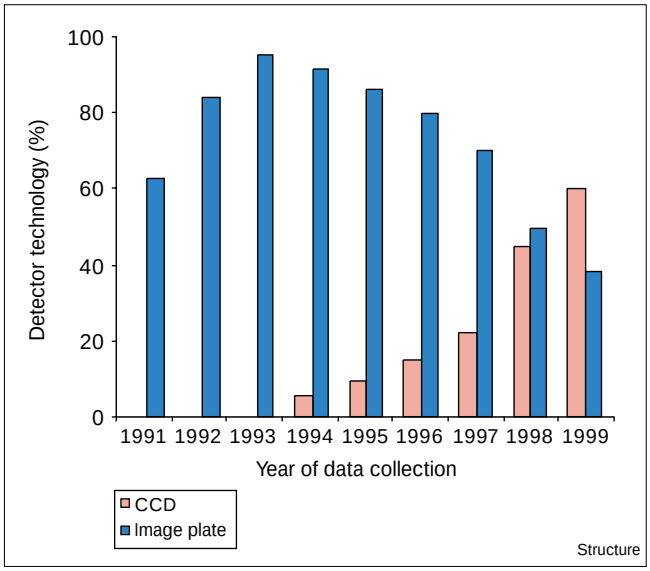
There are numerous advantages of using synchrotron radiation for protein crystallography: rapid data collection, use of smaller crystals than with conventional X-ray sources, and the ability to conduct measurements at multiple wavelengths. The data collection protocols evolved in response to changes in hardware, data integration and phasing software. An experimental protocol is as much a result of a compromise between the various limitations encountered at the beamline as due to the fundamental physics requirements. A typical limitation arises from the difficulty of handling large amounts of data. Currently, the amount of data that can be collected from one frozen crystal can be enormous. A four-wavelength experiment collected with the use of the fast APS-1 detector at the Structural Biology Center (SBC, Argonne National Laboratory) can produce 43 GB of data from a single experiment (180° collected at 0.3° oscillation per frame, 18 MB per frame, four wavelengths) in less than 100 minutes. As shown in this example, the detector's raw ability to measure diffracted X-rays is only one of the limiting factors in synchrotron crystallography. The rate-limiting step is often the ability to back-up and analyze a fast stream of data produced by a multimodule charge-coupled device (CCD). The separation of data acquisition from data backup and analysis makes optimal data collection

Figure 1



The fraction of depositions in the Protein Data Bank (PDB) that reported the use of synchrotron radiation, as of March 25th 2000. Only unique structures are present in these statistics; a unique structure was defined as the set of nonisomorphous structures, where the first reported structure was used.

Figure 2



The fraction of depositions in the PDB that reported the use of synchrotron radiation and a particular detector technology, as of March 25th 2000. Only unique structures and entries that reported the type of detector used were included.

much more difficult, as at many beamlines shuffling data between computers and just editing script files takes more time than the collection of a full data set. The goal of the newly developed HKL2000 package is to integrate all computational activities that have to be performed during the data collection experiment. The graphical command center (GCC) of HKL2000 organizes and forwards the data collection parameters to the display, indexing, strategy, simulation, refinement, integration, scaling and merging tasks. These tasks do not have to be executed on a single computer, and there are plans to extend the GCC to allow the coordination of tasks across a network. The statistical output from these tasks is stored in the project database, and graphical representation can be provided on-line or in the form of the report.

There are two outcomes of diffraction experiments: amplitudes and a phase-sensitive signal that is typically produced by small differences between observed amplitudes. If the goal of the experiment is to collect high-resolution data for refinement or for solution by molecular replacement, the quantity of the data is much more important than its precision, whereas the opposite is true for the measurement of anomalous differences (AD; SAD if a single wavelength is used or MAD if multiwavelength). For the refinement, it is most important to collect a complete data set to the resolution limit of diffraction, whereas for phasing it is most important to collect precise data at lower resolution, as high-resolution data are generally too weak to provide useful phasing signals. These different requirements are one of the reasons for variations in the protocols; the other reasons are the quality of the sample, beam intensity, type of detector, and the integration and phasing software used in the analysis. Because these factors are continuously evolving, the protocols should be constantly reevaluated.

A precise measurement of the X-ray intensity is only an intermediate goal of the diffraction experiment. The ultimate goal is fast and precise measurements of structure-factor amplitudes. The data model that relates X-ray measurements to structure factors contains the unknowns that contribute to the error of the structure factors (absorption, decay, non-uniform exposure, diffuse scattering, etc., plus hardware imperfections). Improving the statistical accuracy of X-ray intensity may increase radiation damage, and will not necessarily result in better estimates of structure factors. Moving the detector away from the crystal will improve the measurement of the individual reflections by increasing the separation from each other and from the X-ray background, but may decrease the number of reflections being measured simultaneously.

The main problem of synchrotron experiments is that an experiment has to be performed in a very short period of time, with the next chance of access being far in the future. To optimize the experiments, one needs to use a series of samples and find the best protocol. Ideally, during one trip one would like to get feedback from the complete analysis of the first crystals used in order to apply it to subsequent crystals. Theoretically, this is feasible today, with strong beams and fast detectors and computers. In practice, decisions are more often made on the basis of prior experience and intuition, which are highly variable factors between experimental groups and projects.

Wavelength optimization

Synchrotron radiation allows a wide range of choice of experimental wavelength. Several factors should be considered when selecting the wavelength for an experiment: the intensity of the beam; absorption in the crystal; detector efficiency; geometric limitations; magnitude of the dispersive effects (anomalous scattering); and air scattering. The radiation dose can be limited by the radiation damage that is characteristic for high flux beamlines or it can be limited by the amount of available data collection time. Radiation damage and scattering power are proportional to $(\text{wavelength})^2$, so the two effects will cancel. At long wavelengths, one has to take into account that scattered photons can be absorbed in a sample. Thus, when the absorption length is smaller than the size of the crystal, the number of scattered photons absorbed increases as the wavelength increases. To put that in perspective, for 300 μm diameter crystals there is a significant increase in absorption of scattered photons above a 2 Å incident wavelength, whereas for 100 μm crystals this threshold is approximately 3 Å.

For wavelengths in the range 0.9–2 Å, both image plates and CCD detectors have high efficiency. The signal is proportional to the energy of the X-rays, however, to a first approximation, the noise is also proportional to the energy so the signal-to-noise ratio is not significantly affected. For each beamline, there is a maximum diffraction angle such that all reflections below that angle can be measured together. There is an obvious advantage to measuring all diffracting reflections, up to the resolution limit. Bragg's law produces the simple formula:

$$\lambda < \frac{\text{resolution}}{2\sin\left(\frac{2\theta}{2}\right)}$$

$$2\theta = \arctan\left(\frac{r}{d}\right)$$

where r is the detector radius and d is the sample-to-detector distance. For example, for a 210×210 mm detector and 120 mm sample-to-detector distance, the maximum value of diffraction angle 2θ is 41.2°. It limits λ to less than the resolution limit × 0.703. Even when the resolution limit is 1.5 Å, wavelengths up to 1.05 Å can be used in an efficient mode of data collection.

There is a small, possibly counter-intuitive, advantage of using long wavelengths to minimize the effect of air scattering. The experiment is affected not by the absolute amount of air scattering, but rather by the ratio of air scatter to crystal scatter. For identical volumes of air and crystal this ratio is not wavelength dependent. In addition, use of the longer wavelength allows one to collect lower resolution reflections at the same beam-stop size and position.

Wavelength optimization should take into account whether the goal of the experiment is phase-sensitive measurement. There are two types of phase-sensitive, wavelength-dependent measurements: Bijvoet differences, which are measured independently at each wavelength, and dispersive differences. Bijvoet differences may be maximized at the absorption edge, but they can also be quite large (or even larger) at wavelengths far from the absorption edge. Dispersive differences are of practical significance near the absorption edge. The signal at the absorption edge is a function not only of atom type, but also its chemical environment. In the case of a selenomethionine (SeMet) anomalous experiment, the impact of oxidation is much larger than any other experimental variable. An oxidized selenium atom has Bijvoet differences up to 50% larger than the reduced form in SeMet. It is possible, and useful, [2] to oxidize selenium in protein crystals to maximize the anomalous and dispersive signals.

Optimal number of wavelengths

The optimal number of wavelengths for a single absorption edge anomalous dispersion experiment is not obvious. In fact, four, three, two and even one wavelength have been proposed as the optimal choice. The suggestion that a single wavelength could be considered comes from the observation that in a four-wavelength experiment, the removal of data collected at the other wavelengths sometimes produced better or equally good quality electron-density maps. This non-intuitive result arises from the non-linearity of the phasing equation and the highly complex error propagation in such a system of equations. The full impact of experimental errors on map quality is outside the scope of this article, but to a first approximation can be described as follows.

The Bijvoet differences have a much higher signal-to-error ratio than dispersive (wavelength-dependent) differences. For this reason most of the useful AD phase information comes from a single-wavelength measurement. In the case of a single kind of heavy-atom contribution, the phase of the heavy-atom substructure does not depend on the wavelength. In such a case, the Bijvoet differences can be effectively averaged between wavelengths with the data measured at the absorption peak having the dominant statistical contribution. The wavelength-dependent differences have much lower statistical significance, but in principle should contribute somewhat to the phase information. Depending on the data collection strategy and radiation damage (even to frozen crystals), there can be some nonisomorphism between different wavelengths. In the case of sulfur and selenium atoms, the heavy-atom structure seems to be particularly sensitive to radiation damage [3]. Nonisomorphism appears to produce more problems than the benefits delivered by the small amount of phase information derived from dispersive differences. The cost-to-benefit ratio of measuring additional wavelengths is quite low in such a case, and it becomes clear that most effort should be spent on optimal data collection at the absorption peak.

Point of diminishing return for phase measurements

As the initial map does not have to be directly interpretable, the experimental requirements change over time as phase improvement procedures advance. In particular, in the past a single source of phase information was considered inadequate for structure solution as it produces an ambiguous phase solution. Nowadays, the phase ambiguity may be resolved by iterative density modification procedures [4].

Phase measurements provide a starting point for phase improvement procedures based on solvent flattening and, where appropriate, maximum entropy and noncrystallographic averaging. The starting phases have to be good enough for the map to be easily (automatically in the best case) interpreted with an atomic model after the phase improvement. With very good quality and high-redundancy data, even the anomalous scattering from sulfur can produce a spectacular experimental electron-density map [5]. In general, a higher fraction of solvent in the crystal lattice strongly increases the ability of solvent-flattening procedures to effectively extend the resolution of the map. For a typical case of approximately 50% solvent, the measured phase signal needs to be significant to at least 3.5 Å resolution. For 70% solvent, even 6 Å starting phases may be enough for structure solution [6]. Noncrystallographic averaging works exceptionally well when the symmetry operator is accurately known, and in several cases with high noncrystallographic symmetry (NCS) (≥ sixfold), averaging and phase extension from very low-resolution globular models have resulted in *ab initio* structure solution. NCS that is not point group symmetry is not usually very exact, and in such a case the amount of phase improvement from averaging cannot be well predicted *a priori*. Maximum entropy and similar methods are still in their early stages of development and may work well in the future, even with a poorer quality set of initial phases than described above.

The phase signal constitutes only a small fraction of the reflection amplitude and therefore has to be measured more precisely. There are two strategies to optimize

measurements: maximize the signal or minimize the error. Synchrotron radiation sources are advantageous for maximizing the phase signal, as a wavelength can be adjusted to an absorption edge so as to minimize quantum statistical error. Other sources of error are minimized by a judicious choice of detector, goniostat, strategy and data analysis method. The purpose of the experiment is to produce a map (phases) that will be subsequently improved by solvent flattening combined with density modification or similar procedures.

Bijvoet differences far from an absorption edge

Between absorption edges, the Bijvoet difference signal increases at a rate proportional to (wavelength)^2. If measurement close to the absorption edge is impractical, it is preferable to measure Bijvoet differences at the longest wavelength that the beamline, crystal absorption and geometrical limitations will allow.

To measure Bijvoet differences, one needs to correct the scale factors for absorption effects. Methods that exploit high data redundancy to reduce systematic errors, such as the ones implemented in SADABS [7] and in HKL2000, allow one to use an optimal wavelength that is much longer than those used in current practice. For sulfur, iodine, xenon, cesium, and many other heavy atoms, the optimal wavelength is typically between 2 Å and 2.5 Å. One can expect large improvements in phasing using these atoms as a source of phase information. At long wavelengths, sulfur-induced Bijvoet differences are likely to produce enough phase information for many protein crystals. An example of an experiment that exploited this new absorption correction was performed at the SBC 19-BM beamline during beamline commissioning. A lysozyme data set was collected with an incident wavelength of 1.7 Å. Table 1 compares the statistics for data to which the absorption correction was both applied and not applied. CNS and Mlphare followed by DM phasing procedures both produced interpretable electron-density maps from this 45 minute experiment.

Long-wavelength crystallography opens the possibility of exploitation of synchrotrons that have the intensity spectrum shifted towards low energies.

Data quality and the experimental goal

Molecular replacement

Success of the molecular replacement method depends more upon the completeness of the data at medium-to-low resolution than on the quality of the data. In particular, the method is less sensitive to systematic multiplicative errors or even large random errors, but is very dependent upon the inclusion of all the strongest reflections. Therefore, it is highly recommended to make an additional low exposure pass to collect low-resolution reflections that may have been 'overloaded' (i.e., owing to saturated detector pixels) in the high exposure, high-resolution data scans.

Table 1

Statistics for merged data with and without semi-empirical absorption correction.

Resolution shell (Å)	Average intensity	σ	R
Absorption correction not applied			
99.00–3.87	69,821.8	1252.5	0.044
3.87–3.07	73,163.7	1213.1	0.048
3.07–2.69	35,357.4	589.7	0.054
2.69–2.44	25,986.8	444.3	0.059
2.44–2.27	21,871.9	390.4	0.061
2.27–2.13	18,007.3	346.4	0.065
2.13–2.02	15,350.2	412.3	0.068
2.02–1.94	11,391.8	420.0	0.073
1.94–1.86	8,161.7	469.5	0.074
1.86–1.80	6,062.3	661.1	0.092
99.00–1.80	32,464.3	638.5	0.052
Absorption correction applied			
99.00–3.87	66,153.5	935.5	0.031
3.87–3.07	70,046.6	735.2	0.029
3.07–2.69	34,150.5	362.2	0.032
2.69–2.44	25,231.9	275.8	0.035
2.44–2.27	21,405.9	245.7	0.037
2.27–2.13	17,774.9	224.8	0.041
2.13–2.02	15,275.8	275.5	0.046
2.02–1.94	11,360.6	316.4	0.053
1.94–1.86	8,050.1	369.9	0.058
1.86–1.80	5,826.5	521.6	0.080
99.00–1.80	31,301.1	431.2	0.033

Refinement

Refinement is sensitive to the completeness of the data and to the high-resolution limit as defined by average values of $I/\sigma \approx 2$ (where I is the diffraction intensity and σ is the error estimate). Refinement is remarkably insensitive to multiplicative errors and redundancy. Detailed discussion of these issues will be presented in the near future (GJ Kleywegt, unpublished).

Isomorphous replacement

In most cases errors owing to chemically induced nonisomorphism are larger than experimental errors, so the multiple isomorphous replacement (MIR) method tends not to be sensitive to the quality of data, except when Bijvoet differences are used for phasing.

Bijvoet and dispersive differences (SAD and MAD)

The phasing power and the quality of the experimental maps are proportional to the ratio of phasing signal-to-noise; measurement errors and radiation damage are the key contributors to the noise level of such experiments. Success depends on careful planning, the use of stable and properly calibrated hardware, and appropriate exposure times that maximize the counting statistics while minimizing radiation damage.

Correlation of errors

The process of finding corrections to known, reproducible errors, where the symmetry-related reflections are placed on the same scale, is called scaling. In practice quite a few corrections have a similar impact on the data and an attempt to fit one error may, in fact, correct for a different one but have a correlated impact on intensities. An important example is given by the error that occurs when the integration area omits part of a reflection. In this case all measurements have a systematic error (intensities are underestimated). If this effect is uniform for all reflections it has no impact for practical protein crystallography, as an absolute scale factor for the data is never estimated. What is important is the variation in the magnitude of the omitted intensity and not the absolute value of the correction.

As an illustration, we analyze the situation where systematic errors are the main source of error in a MAD experiment. Typically, there are many small systematic errors of similar magnitude and, assuming that they are quite different in terms of functional dependence on crystal and instrumental parameters, the proper formula is given by:

$$E^2 = \Sigma e_c^2$$

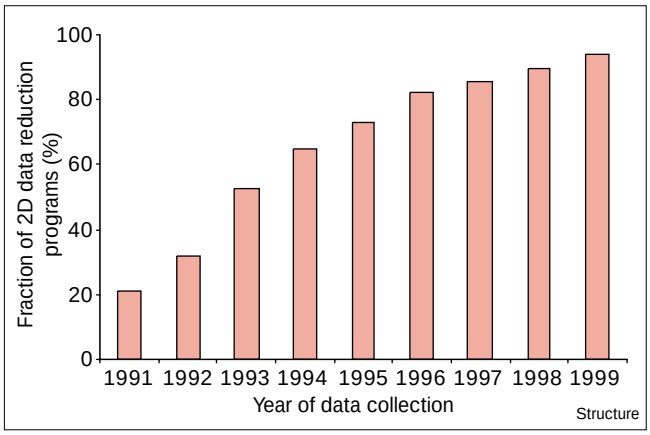
where E is the magnitude of the overall systematic error and values of e_c are the magnitudes of the individual contributors.

In a MAD experiment, the phasing power is proportional to the ratio of the Bijvoet difference signal to the experimental error. Individual elimination of each component of the systematic error has only a very small impact on improving phasing power, discouraging the experimenter from putting too much effort into removing such errors. Because error elimination reduces the denominator of the ratio, however, removing the last component of the error has a dramatic impact on phase and map quality. Removal of the last large source of error always has the most significant impact, whereas elimination of the identical source of error at an early stage results in virtually no impact. This might explain the apparently contradictory statements made by people in the field regarding what is most important for the success of MAD experiments.

2D or not 2D

In addition to the presence of errors, the interaction of errors and error compensation are confounding factors that may obscure the experimental problem. A useful discussion of these topics is presented by Otwinowski and Minor [8] and is given in the HKL program manual [9]. Despite the intuitive superiority of three-dimensional (3D) profile fitting analysis methods, the remarkable success (Figure 3) of data reduction programs that use the two-dimensional (2D) method (2D profile fitting analysis of partial reflections followed by the addition of partials) is due to error compensation during profile fitting analysis [10]. For instance, an imperfect goniostat or slipping crystal makes 3D processing problematic, yet is easily handled through 2D data reduction programs. The ultimate system is capable of handling 2D or 3D data processing as the need arises.

Figure 3



The fraction of PDB entries that reported the use of 2D data reduction programs. Only unique structures and entries that reported the data reduction program used were included. The following programs were classified as 2D: HKL/DENZO [8], Mosflm [11], OSC [12], Rigaku [13] and Weiss.

A primary concern in the use of synchrotron radiation is the measurement of the most difficult (i.e., the weakest) reflections. This can be accomplished by maximizing the total radiation dose delivered to the crystal. An optimization of the detection system for weak reflections would make the overall statistical uncertainty close to optimum. The objective of the experiment (the best possible measurement) is to optimize data completeness and redundancy while taking into account radiation damage to the crystal. The ratio of the total radiation dose to the total oscillation range gives the exposure per degree; this makes the individual frame exposure dose proportional to the frame width. The optimal frame width should be defined by the crystal mosaicity and cell dimensions, but in practice the experiment tends to be designed around the detector and computing resource limitations. Most detrimental to data quality are computing limitations that strongly influence data redundancy and force experimenters to use a wide-angle oscillation data-collection mode. Moreover, at most beamlines the decision about the use of a certain frame oscillation width is based only on an optical inspection of the initial diffraction pattern or, at best, on the information obtained from one frame. CCD detectors allow for very fast data collection, and by collecting a full low-resolution data set a crystal can be completely characterized in a few minutes. This characterization should provide the experimenter with information about the unit cell, crystal symmetry, mosaicity and possible anomalous

signal. A few additional frames can establish the diffraction limit of the sample. The full characterization of a crystal removes the uncertainties involved in the decision of which sample to use and how to measure it — one of the main difficulties synchrotron users encounter.

Future directions

For many projects, the main difficulty is likely to be an inability to grow reasonably well-diffracting crystals. Efficient data collection can reduce the effort needed to grow crystals of sufficient quality for structure solution. At the synchrotron beamline, data collection will become part of a process that includes indexing, integration, scaling and phasing. If the end result is a high-quality electron-density map, it will provide assurance that the experiment was successful and will allow for more effective use of the remainder of the allocated time. This approach will become an essential part of the structural genomics effort.

The increase of Internet bandwidth and in particular the arrival of the Internet-2 will provide an opportunity to remotely interact with the experimental set-up and perform the synchrotron experiment from the home laboratory. Data collection through the Internet will require the automation of sample mounting and alignment, an issue being addressed by a number of groups. Increasingly, elaborate, graphical control programs will coordinate all crystallographic data collection/processing steps. As these programs incorporate more rules, they will become an expert system that leads the experimenter through the whole process.

Acknowledgements

This work was supported by NIH grant GM-53163. The authors would like to thank Zbyszek Dauter for his comments and Andrzej Joachimiak and the SBC staff at Argonne's Advanced Photon Source for their collaboration during synchrotron experiments.

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