Single-Crystal Diffractometry: Strategy for Rapidly Decaying Poorly Diffracting Crystals

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Abstract

A strategy allowing rapid data collection has been developed on a standard Philips PW1100 automatic four-circle diffractometer adapted for protein studies. Hardware modifications include: a long arm enabling a continuous adjustment of the crystal-to-counter distance, a helium pathway with variable apertures on the detector side, and a cooling system for temperatures down to 263 K using an axial flow of dry air blown onto the crystal along the axis. Modifications of the diffractometer software, providing a more efficient data collection strategy, are also described. An integrated set of Fortran programs has been implemented on a 16-bit minicomputer for the processing of the experimental measurements. During the treatment of the Bragg reflections, stored with their profiles, special care is taken for background estimation and decay correction. The set of programs and the strategy have been successfully tested with the structure determinations to high resolution of yeast tRNA\textsubscript{Asp} and of cardiotoxin IV from \textit{Naja mossambica mossambica}. The modified hardware has also been successfully used for data collection of a large number of poorly diffracting small-molecule crystals.

Introduction

Diffraction studies on biological macromolecular crystals can suffer from many burdens. Beside the more classical problems of anisotropic absorption corrections owing to the presence of both the capillary and mother liquor around the crystal and of the important decrease of diffracted intensity with increasing resolution, two other major difficulties are often encountered: the intrinsically poor diffracting power and the time-dependent X-ray induced damages. The first handicap results in a low signal-to-noise ratio, which induces a rapid decay in the diffracted intensities. This low signal-to-noise ratio is not only due to the weakness of the diffracting signal but also to the high background introduced by the air scattering, as shown by Krieger, Chambers, Cristoph, Stroud & Trus (1974). With the structure determination of yeast tRNA\textsubscript{Asp} (Moras et al., 1980), we were faced with both problems: the crystals diffracted to a resolution of 2.7 Å with an average decay of intensity varying from 1% h\textsuperscript{-1} at 3 Å to 0.4% h\textsuperscript{-1} at 10 Å. Although various methods had already been described, based on profile modification (Wyckoff et al., 1967) or profile fitting (Diamond, 1969), none of them was directly usable on our available equipment. This prompted us to design a set of tools and a strategy to optimize the signal-to-noise ratio and the speed of data collection on a Philips PW1100 diffractometer initially designed for small-molecule work. The strategy described here had to take into account more precisely the specific constraints of the data collection of tRNA\textsubscript{Asp}, essentially the resolution of a parameter of 150 Å and the difficulty in reaching the true background. The first step was the adaptation of the goniometer of the diffractometer, the second led to the optimal use of that equipment and the development of software to take advantage of the data collection method. The proposed answer applies to all problems where poor diffracting and/or rapidly decaying crystals exist. The programs, originally written for a Univac 1100 computer were then adapted to a (DEC) PDP 11/60 mini-computer. All the strategy has subsequently been applied to experimental data measured on a Nonius CAD 4 diffractometer.

Diffractometer adaptation

1. Hardware modifications

We report here on the technical adaptations chosen for a Philips PW1100 diffractometer, available in its standard version. Fig. 1 shows the modified diffractometer with the new parts lettered as follows: A: extension arm that holds the detector; B: helium beam tunnel with its collimation; C: beam stop; D: telescope holder; S: location of new collision switches; T: cooling...
nozzle through which the gas stream is blown onto the goniometer head.

Extension arm. To improve the spatial resolution of the diffractometer, it was necessary to increase the crystal-to-detector distance. The arm holding the counter designed by Philips is replaced by a 45 cm long bench made out of duraluminium, bolted to the goniometer through the standard three-point fixation. The new counter holder ($A'$) is mobile to allow a continuous adjustment of the crystal-to-detector distance (15 to 40 cm). When designing this arm, care was taken to lighten all the new parts while keeping the overall stability. The resulting weight (1300 g) compares advantageously with the initial arm (2200 g).

Beam tunnel. To reduce the background, two techniques can be used: either to minimize the volume of air crossed by X-rays by extending the pathway through vacuum or helium, or to reduce mechanically the volume of air that can scatter into the counter. Helium was used by Krieger & Stroud (1976) who developed a gas-flow chamber that provided a drastic improvement. We chose a similar approach although in our set-up the crystal is not bathed in helium.

Reduction of the air scattering of X-rays by the diffracted beam is achieved by a tunnel of variable length adjusted to the selected crystal-to-counter distance. The tunnel is fastened to the detection arm through a holder that is mobile and adjustable in height. Apertures at each end of the tunnel enable a circulation of helium. Replacement of a 17.5 cm path in air (standard geometry) by a 37.5 cm path including 30 cm through helium results in an overall gain in intensity of about 10% of the signal with a concomitant decrease of background due to air scattering. It is possible to mount brass rings of different diameters, allowing the selection of the aperture corresponding to the size and the quality of the crystal under study. The diameters of the rings could vary between 0.2 and 2.0 mm corresponding to an angular aperture of 0.57 to 5.7° when placed 2.0 cm away from the crystal. These apertures, combined with the standard slits located in front of the detector, are the critical parameters that allow the optimization of the signal-to-noise ratio by setting the noise level to its minimum. It must be noted that all the slit widths must be consistent; in particular the angular aperture chosen for the ring close to the crystal must be larger than the maximum angular value corresponding to the slit in front of the counter.

A lead collar, as described later, is set on the helium tunnel to cut the primary beam. It reduces the background observed at 2θ values higher than 18° by stopping the primary beam as close as possible to the crystal, thus considerably limiting the overall X-ray scattering and in particular the scattering that can get into the counter. At lower angles, this effect is obtained by the lead front of the beam tunnel itself. This drastic improvement is shown in Fig. 2 where experimental curves corresponding to the background values are drawn under conditions of full opening of the aperture on the beam tunnel (2 mm) and 3 mm × 1.5 slits on the counter. Under standard data collection conditions with a front aperture of 1 mm and a quartz capillary the background could vary between 8 to 10 counts s⁻¹ at 2θ of 2° to 2 to 30°.

Beam stop. The standard beam stop, fastened to the telescope, presents two major disadvantages: (i) the helium beam tunnel is forced to move back and thus the length of the air path is extended; (ii) being too close to the crystal it also increases the blind area near the origin and suppresses the possibility of collecting useful low-resolution data. A new beam stop, 5 mm thick, was built. It is located between the helium pipe

Fig. 1. (a) General view of the PW 1100 Philips goniometer after hardware modifications; $A$ is the extension arm, $B$ is the helium beam tunnel, $C$ the new beam stop and $D$ the telescope holder adapted for the cooling system. (b) Close up view of the crystal head with $T$ the cooling nozzle through which the gas stream is blown on the goniometer head and $S$ an anticollision safety switch.
DIFFRACTOMETRY OF POORLY DIFFRACTING CRYSTALS

and the detector, on the primary beam path. It is bolted to the frame of the goniometer using two existing screws. Its position can be adjusted to adapt to the location of the detector and of the helium tunnel. The size of the lead disc, fixed to a brass holder, enables one to collect diffracted data below a Bragg angle of 1°.

As a consequence of the new location of the beam stop, the path length of the primary beam in air is largely increased, thus producing an important air scattering of X-rays. To cure this problem, a lead collar was fixed to the helium pipe near the crystal, which intercepts the primary beam up to a 2θ angle of 18° as shown in Fig. 2.

Safety switches. A direct consequence of the presence of the helium beam tunnel is a limitation of the relative moments of the goniometer around the ω and 2θ circles. To avoid collisions that would result in hardware damage and misalignments, it is necessary to introduce new collision sensors activated by electrical contacts. Two sensitive parts of the goniometer have been protected: sensor S1 stops the diffractometer in case of a collision resulting from an ill-timed ω–2θ motion, S2 avoids the contact between the cooling system and the helium pipe in case of errors in programmed limits or errors in manipulation under manual control.

2. Software modifications

The software of the PW11000 diffractometer had to be modified for two reasons: (1) to allow for the hardware developments; (2) to adapt the programs to the problems tackled: increased data collection speed and improvement of the signal-to-noise ratio.

The major software modifications, however, deal with the data collection procedure. They can easily be adapted on any Fortran version of the PW1100 diffractometer software. They were actually performed on a P852M Philips mini-computer (16 bits, 32 K memory) equipped with two Philips P833 cassette drives for mass memory and a P831 magnetic tape drive.

The first software modification to be implemented had to allow for the possibility of modifying the crystal-to-detector distance. This value is used to compute the angular steps for the movement of the four circles during the centering of a reflection and during the peak-hunting procedure. The ratio of the standard distance to the new distance is now a variable to be input.

First, the content of a table containing the variables used in the data collection (orientation matrix, speed, scan width, indices of the standard reflections) is written on the magnetic tape. This is now automatically done when the instruction SDC (start data collection) is typed. This facility was considered to be very useful since it minimizes the book keeping associated with each data collection especially when modifications occur during a set of measurements and prevents errors in further data treatments.

The procedure used for data collection is the flying step-scan. During a scan, the number of recorded counts is unloaded every fraction of time, giving then a measurement of the number of counts corresponding to each step. For a given scan width, the only degree of freedom is the fraction of time that induces the number of steps. This would normally result in a variable length of the record for each reflection since the scan width is usually related to resolution. In fact, for convenience in further treatments, the length of the records for each hkl written on tape was standardized to 240 characters corresponding to a maximum of 30 steps.

To take advantage of the profile analysis procedure, the full profiles are written on tape for each measurement of the standard reflections used to monitor the data collection and the decay corrections.

To save time during data collection, a modification of the generation of indices was also introduced. The procedure was modified in order to avoid generating non-used indices (for example, systematic extinctions and hkl corresponding to a portion of the reciprocal space that was not going to be measured). As a result, in the case of a crystal with a large unit cell like tRNAAsp belonging to the space group C2221, this simple modification save up to 20% of the time spent in data collection.
The possibility of reading indices of reflections to be measured from an external device was also added. In such a case, the diffractometer is driven using indices of reflections that had previously been copied to a cassette. This facility of a master file was used to measure the same set of reflections at the beginning and at the end of the data collection and for anomalous data measurements. In this last case, we measure sets of up to 49 reflections followed by the related Friedel equivalents, thus minimizing the time spent in moving circles.

Finally, an additional option was introduced in the scanning procedure, the control of the crystal centering on reflections of intensities larger than a given adjustable level. If the step of maximum intensity deviates from the center of the scan by more than a chosen number of steps, the crystal is then automatically recentered.

Data collection

1. Strategy

The first question to be answered when a crystal is oriented is the limit of resolution to be reached during data collection. This can be decided on the basis of the diffracting power, lack of isomorphism or other criteria. When very few strongly diffracting crystals can be obtained, this choice is essential and needs to be made carefully in order not to miss any opportunity to collect high-resolution data. This was especially true in the case of the tRNAAsp crystal. Once the decision is taken, the reciprocal space to be investigated is divided into shells of equal volume and the data collection proceeds from the outside shell toward the center of reciprocal space. This procedure presents the advantage of measuring first the high-resolution weak reflections with a reasonable decay. Furthermore, for a given range of resolution, the data are all measured within a short period of time.

Assuming a rate of measurement, the next decision to be taken is the desired redundancy of data from a given crystal. If crystals have a short lifetime under X-ray exposure, many of them are needed anyway for a complete data set, thus we choose to measure each reflection several times (at least three) on different crystals and accept a larger decay. This approach gives a better image of the diffracted intensities of the compound, since it integrates the dispersion of measurements from different crystals and facilitates the scaling of the data. If the lifetime of the crystals is sufficient for collecting a full data set, crystallographically equivalent reflections are recorded on the same crystal. Furthermore, at the end of the data collection, a set of starting reflections is measured again in order to determine the decay correction factors.

Usually the best diffracting crystal is used for a systematic scan of the reciprocal space at a reasonable speed (0.032° s⁻¹). The resulting significant reflections are used to set up 'master files' on disk or tape. These files are used to collect data with smaller crystals and for Friedel pair measurements. In the latter case, reflections are collected by blocks of 49 neighboring hkl followed by 49hkl, to minimize time spent in displacements of the goniometer head.

A typical data collection starts with the measurement of 50 scaling reflections evenly distributed in the reciprocal space, followed by the scan of the desired sets of hkl and finishes with a new set of scaling reflections. Three standard reflections are used to monitor the crystal alignment. For each hkl, the scan width is usually three times the half width of the peak.

2. Data processing

The various steps involved are summarized on the flow chart of Fig. 3. The experimental data are stored on a magnetic tape or a disk file called DIFDAT (for diffractometer data). The content of the file begins with all items of crystallographic information concerning the crystal and the various parameters of the data collection (orientation matrix, scan width, step width, speed, standard reflections,...); the individual measurements (scaling reflections, data set) follow. This file is used as an input file to the data reduction program (DATARED). Analysis of the experimental data is done by this program and proceeds in two consecutive runs. In the first pass, the program prints some selected profiles for visual inspection, sets up a background table and finds the decay parameters. These constants are then applied to the measured intensities in a second pass, which is the actual data reduction step.

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Background determination. All measurements are analyzed for background determination. Each profile consists of approximately 15 steps of equal width. The step with the lowest count is discarded. This is to take account of a possible incomplete step at the end of a scan, a phenomenon that can occur using flying step-scan techniques. The steps with the next lowest counts, two to four depending on the scan profile, are averaged and this value is stored in a two entries table \((\theta, \varphi)\), the resolution \((\theta)\) and the position of the crystal with respect to the primary beam \((\varphi)\). Two functions \(f(\theta)\) and \(g(\varphi)\) are then computed, the product of which \([f(\theta)g(\varphi)]\) provides a good and reliable approximation of the true background as a function of \(\theta\) and \(\varphi\). This last variable has been found very useful in the case of crystals with asymmetric shape. \(f(\theta)\) is the weighted averaged background value in a given \(\theta\) range for the different \(\varphi\) angles where data have been collected, \(g(\varphi)\) is the normalized weighted averaged ratio (background/background maximum) in a given \(\varphi\) range for the different values of \(\theta\) where data were collected.

Owing to narrow scans, the background level is not reached for the highest diffracted intensities. To avoid such a bias, these reflections are rejected from background statistics through a cut-off value, which is an input parameter of the program. Statistics are printed to detect misbehavior in the results. The averaged values of the background per step \((B_k)\) are then stored for the second pass.

Profile-fitting approach. Numerous solutions to the profile-fitting approach have been proposed (Hanson, Wartenpaugh, Sieker & Jensen, 1979; Lehmann & Larson, 1974; Blessing, Coppens & Becker, 1974; Tickle, 1975; Birknes & Hansen, 1983). We tested one of these methods on our data (Lehmann & Larsen, 1974) but this attempt was unsuccessful owing to the large cell parameter of tRNA\(^{\text{Asp}}\) (150 Å), which prevents the true background of the medium and strong reflections being obtained, and, more generally, to the anisotropic profile of the diffracted intensities. This forced us to use the more elementary approach described here. A profile analysis is performed for each reflection. In order to optimize the centering of the peak and the signal-to-noise ratio a window of \((2N + 1)\) steps, \(N\) being an input parameter, is moved from the beginning of the scan to its end. The highest count value of the run of the \((2N + 1)\) contiguous steps is kept as the raw intensity of the reflection. The net intensity is given by the relation:

\[
I_N = I_{\text{raw}} - (2N + 1) B_k \]

\[
\delta(I) = [I_{\text{raw}} + (2N + 1^2 B_k)]^{1/2}. \]

This procedure, which consists of searching for the maximum value of \((2N + 1)\) consecutive steps, is comparable to the method first introduced by Watson, Shotton, Cox & Muirhead (1970). There are two main differences: only slight peak missetings are accepted (usually no more than two steps, typically 0.05 or 0.10°) and background is extracted from the previously defined table instead of being taken from the same scan.

Decay corrections. For tRNA\(^{\text{Asp}}\), the decay of intensities was observed to be very sensitive not only to time but also to resolution (i.e. 0.4% h\(^{-1}\) at 10 Å resolution and 1% h\(^{-1}\) at 3 Å). Therefore, in the decay correction both effects were included according to the expression:

\[
I_{\text{corr}} = I_{\text{obs}}(y_0/y) \]

\[
y = y_0(1 + b S) \exp(B \sin^2 \theta/\lambda^2), \]

where \(S\) is the sequence number of the reflection, \(b\) and \(B\) are the decay coefficients. The factor \(b\) is obtained from a least-squares analysis of the three standard reflections. \(B\) accounts for the resolution dependence and is given by a least-squares fit between the scaling reflections measured at the end of the data collection and the same set measured at the beginning.

Net intensities are then corrected for absorption effects using an empirical curve as described by North, Phillips & Matthews (1968) and for Lorentz and polarization factors. The procedure used takes into account both \(\theta\) and \(\varphi\) angles as shown in Table 1. An array with the background values as a function of \(\theta\) and \(\varphi\) is first produced as previously described; two functions \(f(\theta)\) and \(g(\varphi)\) are then computed from the value of the background along the rows and the columns of the array. For a given pair of \(\theta\) and \(\varphi\) values, the background is obtained as the product of \(f(\theta)\) by \(g(\varphi)\).

Averaging and fitting. The reduced data are sorted according to \(hkl\) and equivalent reflections are averaged. The averaging statistics are printed in a two entries table \((\sin \theta/\lambda \text{ and } F_{\text{obs}})\), which is used as an additional check of the data reduction process. The resulting unique data set is then fitted to a common native file with program FIT adapted from a program originally written by M. G. Rossmann and described by Adams et al. (1969). The outputted new FIT file can be used in the Enraf–Nonius SDP package (Frenz, 1978), or in any local library.

All programs are written in Fortran for a 16 bit disk oriented minicomputer and are available upon request from the authors.

Results

The data collection strategy described here, including the hardware developments for the diffractometer and all the software developments, was successfully applied to the structure determination of two molecules: tRNA\(^{\text{Asp}}\) from yeast (in fact two non-isomorphous
Table 1. Values of the background as a function of \( \theta \) and crystal orientation along the \( \phi \) axis derived from preprocessing a crystal data set, \( f(\theta) \) derived by a weighted averaging as a function of resolution and average weighted ratio \( g(\phi) \) (background/background maximum) as a function of \( \phi \)

<table>
<thead>
<tr>
<th>( \theta(\degree)/\phi(\degree) )</th>
<th>9-9.5</th>
<th>9.5-10</th>
<th>10-10.5</th>
<th>10-11</th>
<th>11-11.5</th>
<th>11.5-12</th>
<th>12-12.5</th>
<th>12.5-13</th>
<th>13-13.5</th>
<th>13.5-14</th>
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<td>72-84</td>
<td>29.3</td>
<td>29.8</td>
<td>31.5</td>
<td>37.4</td>
<td>44.2</td>
<td>47.2</td>
<td>46.5</td>
<td>46.2</td>
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<td>1.1</td>
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<td>108-120</td>
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<tr>
<td>120-132</td>
<td>1.1</td>
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<tr>
<td>156-168</td>
<td>1.1</td>
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</table>

Table 2. Crystallographic parameters of the three structures for which data were recorded on the PW 1100 Philips diffractometer and are now solved: yeast tRNA\(^{A_{SP}}\) in two crystal forms (Moras et al., 1980) and cardiotoxin IV from Naja mossambica mossambica (manuscript in preparation)

<table>
<thead>
<tr>
<th></th>
<th>Space group</th>
<th>( a (\text{Å}) )</th>
<th>( b (\text{Å}) )</th>
<th>( c (\text{Å}) )</th>
<th>( V (\text{Å}^3) )</th>
<th>Resolution to which data were recorded on the diffractometer (Å)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Yeast tRNA(^{A_{SP}})</td>
<td>C 222(_{1})</td>
<td>61.5</td>
<td>67.5</td>
<td>149.5</td>
<td>620.612</td>
<td>3.0</td>
</tr>
<tr>
<td>crystal form A</td>
<td></td>
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<tr>
<td>Yeast tRNA(^{A_{SP}})</td>
<td>C 222(_{1})</td>
<td>60.3</td>
<td>68.0</td>
<td>149.5</td>
<td>618.093</td>
<td>2.8</td>
</tr>
<tr>
<td>crystal form B</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Snake cardiotoxin</td>
<td>P6(_{1})</td>
<td>73.8</td>
<td>73.8</td>
<td>58.9</td>
<td>277.951</td>
<td>2.7</td>
</tr>
</tbody>
</table>

Crystal forms) and a cardiotoxin from Naja mossambica mossambica. All structures have been solved, the tRNA to a resolution of 3.0 Å (crystal form A) and 2.8 Å (crystal form B) (Moras et al., 1980) and the cardiotoxin to a resolution of 2.7 Å. The cell parameters of the different crystal forms are summarized in Table 2. Parts of these molecules are still under refinement. At the present stage, the structure of yeast tRNA\(^{A_{SP}}\) (form B) has been refined to an \( R \) value of 23% while form A is still under refinement (present \( R \) 27%). This is a posteriori evidence of the accuracy of the data used to solve the structures.

We would like to thank Professor M. G. Rossmann for supplying the fitting program and Dr B. Rees for the original version of the SORT program and many helpful discussions. We are also indebted to Dr A. Mitchler and J. Lenz for contributions to the design.
and the skillful execution of the cooling nozzle. The extension arm was made by the central workshop of CNRS at Cronenbourg (Strasbourg).

References


