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X-Ray Crystallography of Chemical Compounds

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Abstract

Aims—Accurate knowledge of molecular structure is a prerequisite for rational drug design. This review examines the role of x-ray crystallography in providing the required structural information and advances in the field of x-ray crystallography that enhance or expand its role.

Main Methods—X-ray crystallography of new drugs candidates and intermediates can provide valuable information of new syntheses and parameters for quantitative structure activity relationships (QSAR).

Key Findings—Crystallographic studies play a vital role in many disciplines including materials science, chemistry, pharmacology, and molecular biology. X-ray crystallography is the most comprehensive technique available to determine molecular structure. A requirement for the high accuracy of crystallographic structures is that a ‘good crystal’ must be found, and this is often the rate-limiting step. In the past three decades developments in detectors, increases in computer power, and powerful graphics capabilities have contributed to a dramatic increase in the number of materials characterized by x-ray crystallography. More recently the advent of high-throughput crystallization techniques has enhanced our ability to produce that one good crystal required for crystallographic analysis.

Significance—Continuing advances in all phases of a crystallographic study have expanded the ranges of samples which can be analyzed by x-ray crystallography to include larger molecules, smaller or weakly diffracting crystals, and twinned crystals.

Keywords

x-ray crystallography; drug design; absolute configuration

Introduction

In the world of biologically active molecules structure and function are intimately related (Griffin and Duax, 1982). The enantioselectivity of an enzyme is directly related to its chirality, thus methods which can determine the chirality (or absolute configuration) of a molecule are key in the studies of such molecules. X-ray crystallography is the primary method for determining the absolute configuration of a molecule and results from crystallographic studies provide unambiguous, accurate, and reliable 3-dimensional structural parameters, which are prerequisites for rational drug design and structure-based functional studies. Thus the role of

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crystallography in drug design is widely recognized (Cachau and Podjarny, 2005; Deschamps, 2005; Williams et al., 2005; Blundell et al., 2002; Wouters and Ooms, 2001).

There are four major steps involved in an x-ray study: crystallization, data collection, structure solution, and refinement/validation. In each of the past three decades technology has produced major advances in three of the four phases of a crystallographic analysis while in the last decade we have begun to see major advances in crystallization. Data collection techniques have progressed from collecting reflections on photographic films that required manual estimation of intensity through automatic diffractometers using photomultiplier tubes to measure one reflection at a time to the current state of the art using two-dimensional area detectors capable of measuring hundreds of reflections at once. These advances have reduced the time needed for data collections from weeks to days. In parallel with this improvements in x-ray generators have produced laboratory x-ray sources with increased brilliance which has also contributed to reduced data collection times. Equally impressive advances in computer hardware and software development have reduced the time needed to solve and refine a structure from days or weeks to hours. In combination these advances have enabled crystallographic studies of both very small crystals and very large molecules. Thus tasks that once required months of effort can now be accomplished in hours. The review that follows describes the fundamentals of a crystallographic experiment and illustrates the effects of these improvements.

Crystallization

The process of x-ray crystallography starts with the crystallization of a molecule of interest. Crystal growth experiments are still done in much the same way that they were done 30 years ago and have become the rate determining step in many crystallographic studies. All strategies for crystallization are aimed at bringing a concentrated solution of a homogeneous population of molecules very slowly toward a state of minimum solubility. The goal is to achieve a limited degree of super-saturation, from which the system can relax by formation of a crystalline precipitate. Crystallization can take on many forms and is often dictated by the type of molecule being crystallized. In general crystallization is accomplished by mixing the sample with a variety of different chemicals that may cause it to crystallize, or by slowly concentrating a solution of the sample (Figure 1). Many techniques developed for achieving these ends have been described (McPherson, 1982; Etter et al., 1986). For example, the crystallization of a small molecule is often accomplished by evaporation from an organic solvent (slowly concentrating the sample) while the crystallization of a protein is often accomplished by micro-vapor diffusion using other chemicals as precipitants that reduce the solubility of the sample.

The methods available for crystallization are as varied as the molecules being crystallized and include evaporation and micro-vapor diffusion from the examples above as well as bulk, batch, dialysis, and free interface diffusion methods. For some of these techniques 'micro' versions are available that allow a large number of experiments to be done on a small amount of sample. While an evaporation experiment is relatively simple with only a few variables such as the choice of solvent, temperature, and rate of evaporation, other techniques may have many more variables. When crystallizing a protein the initial sample concentration, drop (or experiment) size, pH, ionic strength, the nature of the precipitant, temperature, and cofactors or counter ions can all be varied in an attempt to grow a diffraction quality crystal. With so many possible variables it is obvious why micro-crystallization techniques are used. This still leaves the investigator with a daunting range of conditions to explore. As a result of this some labs have turned to high-throughput screening methods to determine initial crystallization conditions. In high-throughput methods both the experimental set up (Luft et al., 2003) and documentation and scoring of the results (Cumba et al., 2003) are automated allowing a wide range of conditions to be examining with minimal effort. Initial screens in high-throughput experiments

generally include 1500 or more trials under widely varying conditions increasing the chances of quickly finding suitable crystallization conditions.

The goal of any crystallization experiment is to produce single crystals suitable for x-ray diffraction. Not all experiments result in crystal formation and not all crystals are suitable for x-ray diffraction (Figure 2). Some compounds crystallize as thin plates which often stick together, or worse yet form stacks that appear to be single. Rapid crystal growth can sometimes result in many crystals growing from a single nucleation center – this is not uncommon when the crystals are elongated or needle shaped. Harvesting these needles can produce structures resembling glass wool or dust balls. In many of these cases a single crystal can be isolated from one of these unsuitable forms by careful dissection.

Data Collection

The first experiments in x-ray diffraction were recorded on film and this was the main method of collecting x-ray diffraction data until the 1970s. Automated diffractometers were first available in about 1955 and by the mid 1970s over 1000 structures were added to the Cambridge Structure Database (CSD) each year with data for over 80% of the new structures collected on an automated diffractometer (Deschamps and Flippen-Anderson, 2001). Improvements in data collection continued with the early area detectors becoming available in the 1980's with continuing improvements in the speed and sensitivity of these since then (Deschamps and George, 2003).

In parallel with the improvements in detectors there have also been improvements in x-ray sources (Table 1). For laboratory sources these advances have resulted in approximately a 167 fold increase in incident beam intensity compared to a conventional sealed x-ray tube. Data collection on a beamline at a synchrotron would yield approximately a 20 fold increase in incident beam intensity over the best laboratory source.

One example of the effect of these improvements can be found in the study of 4,4,8,8-Tetranitro-2,6-dioxabicyclo[3.3.0]octane (TNBO). When first studied in the mid-1980s the crystal structure had two molecules in the asymmetric unit, one of which was severely disordered. When crystals of TNBO were re-examined in 2002 a different unit cell was found (Flippen-Anderson et al., 2002). The new cell had a volume three times larger than that of the original cell. In this 'super' cell, two thirds of the data were quite weak, but too strong to be ignored. The new results revealed a structure with six molecules in the asymmetric unit with no disorder; five of the six unique molecules are essentially identical. A regular pattern of strong and weak data can be seen in the 2002 data. From an understanding of this pattern and the relationship between the new larger cell and the original cell it was possible to calculate the average intensities for both the strong and weak data within each layer of the large cell. The strong data are approximately eight times more intense than the weak data. When combined with the non-trivial transform required to move from the small to large cell it is not surprising that the larger cell went undiscovered in 1984. Thus this simple molecule presents an interesting case study to show how much data-collection hardware and software have improved since the middle-1980s.

Absolute Configuration

Friedel's law states that the scattering from the front and back of a plane, *hkl*, is the same. This means that the measured intensities of the 111 and -1-1-1 reflections (and all other 'Friedel' pairs) should be equivalent. However, in a 1930 study on ZnS by Coster, Knol, and Prins it was noted that the 111 reflection was not equivalent to its Friedel mate. This was the first example of anomalous scattering. The difference due to anomalous scattering is greatest when data are collected near an absorption edge of a heavy atom in the structure. It was nearly 20

years later that Bijvoet realized this principle could be used to determine the absolute configuration of the sodium rubidium salt of (+)-tartaric acid.

In general determination of absolute configuration requires a heavy atom in the structure (Figure 3). Alternatively inclusion of a salt of known chirality can be used to set the hand, or a known inverting chiral center in the molecule can be used to set the handedness of the crystal structure. With the advent of area detectors crystallographers are now collecting more 'redundant' data. If none of the preceding conditions are met it is still possible to determine the relative configuration of multiple chiral centers in the same crystal structure. In this case you can only determine if a pair of centers has the same configuration or opposite configurations (i.e. R,S and S,R are equivalent as are R,R and S,S).

A commonly used metric in the determination of absolute configuration is the Flack parameter (Flack, 1983). In the computation of the Flack parameter a crystal is treated as if it is twinned by inversion and the occupancies of the two domains calculated. Physically meaningful values of the Flack parameter range from 0 to 1, although experimental values may lie outside of this range due to statistical fluctuations and systematic errors. For a crystal of an enantiomerically pure compound in the correct absolute configuration the value of the Flack parameter is zero, if the structure is inverted the value is 1. An excellent review of the use and limitations of x-ray crystallography to determine absolute configuration was recently published (Flack and Bernardinelli, 2008). Despite some difficulties, and possible systematic errors, it may be possible to determine the absolute configuration from a 'light atom' (i.e. C, N, and O) structure using highly redundant data (Parsons, 2002).

Summary

Advances in x-ray generators and detectors in combination with improvements in computer hardware and software have greatly improved our ability to analyze crystals that are not of optimal size or shape, and have allowed analysis of very large complexes. These advances are essential to the success of structural studies on endogenous biomaterials. The snapshot provided by x-ray diffraction studies provides information on chemical connectivity, absolute configuration (when conditions are correct), and interactions of biomolecules. Improvements in methods may allow the determination of absolute configuration from a 'light atom' structures.

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Figure 1.

Common experimental set ups for crystals growth. Crystals are often grown by evaporation (left) the results of which can be improved reducing the rate of evaporation by covering the sample. In some cases vapour diffusion (center) is useful in which case the sample is dissolved in one solvent and placed in the inner vial while a second solvent is placed in the outer vial. The experiment is sealed and allowed to come to equilibrium. By carefully selecting the solvents a non-solvent can slowly be diffused into the sample to cause it to become insoluble. Hanging-drop micro-vapour diffusion (right) is often used for samples soluble in aqueous solutions. In this type of experiment a 1 μ l to 5 μ l drop of sample is mixed with an equal volume of precipitant and the drop suspended over a reservoir of the precipitant. As the droplet comes to equilibrium its volume decreases with both the sample and precipitant concentrations increasing to drive the sample out of solution and form crystals.

Figure 2.

Crystal growth is often a rate limiting step in a crystallographic study and not all crystals are suitable for x-ray diffraction. Plates are often stacked (top left) and in this form may not produce useful x-ray diffraction data, but careful dissection of such agglomerations can yield useful single crystals (bottom left). In some experiments rapid growth results in many crystals growing from a common nucleation centre (top middle). These can be separated to yield useful single crystals (bottom middle). Among the least desirable crystal forms are fine needles (top right) that in the can resemble glass wool. Even these can yield useful crystals (bottom right) when diffracted on a state of the art laboratory system where crystals as small as $5 \times 40 \times 220$ microns can be used for data collection (Francisco et al., 2003).

Figure 3.

Determination of absolute configuration places specific constraints on the crystallographic experiment. A heavy atom (top left asterisk, Parrish et al., 2004), reference molecule (top right asterisk, Matecka et al., 1994), or known inverting chiral center (bottom left asterisk, Chen et al., 2003) must be present for determination of absolute configuration. Only the relative configuration of multiple chiral centers (bottom right, asterisk for each chiral center) can be determined if at least one of the preceding conditions is not met. The molecules depicted are: 7 β -Chloro-6,14-*endo*-etheno-6,7,8,14-tetrahydrothebaine (top left), bis(2R,5S-(-)-2,5-Dimethyl-4-(2-(bis(4-fluorophenyl)methoxy) ethyl)piperazinium) co-crystallized with O,O'-dibenzoyl-L-tartrate (top right), 7a-Acetyl-4,5a-epoxy-3,6-dimethoxy-5b-butyl-17-methyl-6a,14a-ethenoisomorphinan (lower left), and (1R,3S)-ethyl 1-(4-methoxyphenyl)-2,3,4,9-tetrahydro-1H-pyrido[3,4-b]indole-3-carboxylate (note may also be 1S,3R since this is a relative conformation).

Table 1

Relative intensity and brilliance of x-ray sources.

X-RAY SOURCE	RELATIVE INTENSITY	APPROXIMATE BRILLIANCE²
Conventional Sealed Tube ¹	1	0.6×10 ⁹
Microfocus Sealed Tube ¹	6	3.6×10 ⁹
Early Rotating Anode ¹	7	4.4×10 ⁹
Microfocus Rotating Anode ¹	37	2.2×10 ¹⁰
Microfocus Rotating Anode/w Advanced Optics ¹	117	7.0×10 ¹⁰
High-Brilliance Rotating Anode/w Advanced Optics ¹	167	1.0×10 ¹¹
Synchrotron (APS 14-ID-B)	3173	1.9×10 ¹²

¹ Data from industry publications² photons/mm²/sec