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18.4. Refinement at atomic resolution

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18.4.1. Definition of atomic resolution

X-rays are diffracted by the electrons that are distributed around the atomic nuclei, and the result of an X-ray crystallographic study is the derived three-dimensional electron-density distribution in the unit cell of the crystal. The elegant simplicity and power of X-ray crystallography arise from the fact that molecular structures are composed of discrete atoms that can be treated as spherically symmetric in the usual approximation. This property places such strong restraints on the Fourier transform of the crystal structures of small molecules that the phase problem can be solved by knowledge of the amplitudes alone.

Each atom or ion can be described by up to eleven parameters (Table 18.4.1.1).

The first parameter is the scattering-factor amplitude for the chemical nature of the atom in question, computed and tabulated for all atom types [*International Tables for Crystallography*, Volume C (1999)]. Once the chemical identity of the atom is established, this parameter is fixed.

The next three parameters relate to the positional coordinates of the atom with respect to the origin of the unit cell.

At atomic resolution, six anisotropic atomic displacement parameters are used to describe the distribution of the atoms in different unit cells (Fig. 18.4.1.1). Atomic displacement parameters (ADPs) reflect both the thermal vibration of atoms about the mean position as a function of time (dynamic disorder) and the variation of positions between different unit cells of the crystal arising from its imperfection (static disorder). Contributors to the apparent ADP ($U_{\rm atom}$) can be thought of as follows (Murshudov *et al.*, 1999):

$$U_{\rm atom} = U_{\rm crystal} + U_{\rm TLS} + U_{\rm torsion} + U_{\rm bond}, \qquad (18.4.1.1)$$

where $U_{\rm crystal}$ represents the fact that a crystal itself is generally an anisotropic field that will result in the intensity falling off in an anisotropic manner, $U_{\rm TLS}$ represents a translation/libration/screw (TLS), *i.e.* the overall motion of molecules or domains (Schomaker & Trueblood, 1968), $U_{\rm torsion}$ is the oscillation along torsion angles and $U_{\rm bond}$ is the oscillation along and across bonds. In principle, all these contributors are highly correlated and it is difficult to separate them from one another. Nevertheless, an understanding of how $U_{\rm atom}$ is a sum of these different components makes it possible to apply atomic anisotropy parameters at different resolutions in a different manner. For example, $U_{\rm crystal} + U_{\rm TLS}$ can be applied at any resolution, as their refinement increases the number of parameters by at most five for $U_{\rm crystal}$ and twenty per independent moiety for $U_{\rm TLS}$. In contrast, refinement of the third contributor does pose a problem, as there is a strong correlation between different torsion angles. As an alternative, ADPs along the internal degrees of freedom could in principle be refined. The fourth and final contributor, $U_{\rm bond}$, can only be refined at very high resolution. In real applications, $U_{\rm crystal}$ and $U_{\rm TLS}$ are separated for convenient description of the system, but in practice their effect is indistinguishable.

In the special case when the tensor $U_{\rm atom}$ is isotropic, *i.e.*, all non-diagonal elements are equal to zero and all diagonal terms are equal to each other, then the atom itself appears to be isotropic and its ADP can be described using only one parameter, $U_{\rm iso}$.

Thus for a full description of a crystal structure in which all atoms only occupy a single site, nine parameters must be determined: three positional parameters and six anisotropic ADPs. This assumes that the spherical-atom approximation applies and ignores the so-called deformation density resulting from the non-spherical nature of the outer atomic and molecular orbitals involved in the chemistry of the atom (Coppens, 1997).

For disordered regions or features, where atoms can be distributed over two or more identifiable sites, the occupancy introduces a tenth variable for each atom. In many cases, the fractional occupancies are not all independent, but are constant for sets of covalently or hydrogen-bonded atoms or for those in non-overlapping solvent networks. This would apply, for example, to partially occupied ligands or side chains with two conformations.

Thus, at atomic resolution, minimization of the discrepancy between the experimentally determined amplitudes or intensities of the Bragg reflections and those calculated from the atomic model requires refinement of, at most, ten (usually nine) independent parameters per atom. This has been achieved classically by least squares, as described in *IT* C (1999), or more recently by maximum-likelihood procedures (Bricogne & Irwin, 1996; Pannu & Read, 1996; Murshudov *et al.*, 1997).

Atomicity is the great simplifying feature of crystallography in terms of structure solution and refinement. If atomic resolution is achieved, there are sufficient accurately measured observables to refine a full atomic model for the ordered part of the structure, but this condition can only be defined somewhat subjectively. A

Table 18.4.1.1. *The parameters of an atomic model*

Parameter type	Number	Variable or fixed
Atom type	1	Fixed after identification
Positional (x, y, z)	3	Variable, subject to restraints
ADPs:		
isotropic	1	Variable beyond about 2.5 Å
anisotropic	6	Variable beyond about 1.5 Å
Occupancy	1	Variable for visible disorder

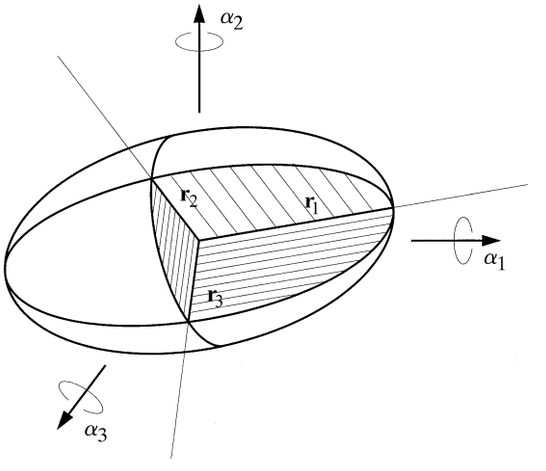

Fig. 18.4.1.1. The thermal-ellipsoid model used to represent anisotropic atomic displacement, with major axes indicated. The ellipsoid is drawn with a specified probability of finding an atom inside its contour. Six parameters are necessary to describe the ellipsoid: three represent the dimensions of the major axes and three the orientation of these axes. These six parameters are expressed in terms of a symmetric U tensor and contribute to atomic scattering through the term $\exp[-2\pi^2(U_{11}h^2a^{*2} + U_{22}k^2b^{*2} + U_{33}l^2c^{*2} + 2U_{12}hka^*b^* \cos\gamma^* + 2U_{13}hla^*c^* \cos\beta^* + 2U_{23}klb^*c^* \cos\alpha^*)]$.

Table 18.4.1.2. *Features which can be seen in the electron density at different resolutions*

Disordered regions will not necessarily be visible even at these limiting values. Some features should be included even at lower resolutions, *e.g.* hydrogen atoms at their riding positions can be incorporated at 2.0 Å, but their positions will not be verifiable from the density. The contents of this table should not be taken as dogmatic rules, but as approximate guidelines.

Resolution (Å)	Feature
1.5	Hydrogen atoms, anisotropic atomic displacement
2.0	Multiple conformations
2.5	Individual isotropic atomic displacement
3.5	Overall temperature factor
4.0	α-Helices and β-sheets
6.0	Domain envelopes

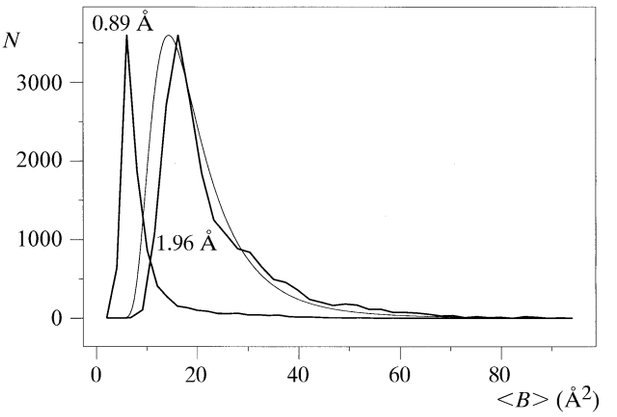

Fig. 18.4.1.2. Histograms of B values for a protein structure, *Micrococcus lysodecticus* catalase (Murshudov *et al.*, 1999), for two different crystals which diffracted to different limiting resolutions. For both crystals, the resolution cutoff reflects the real diffraction limit from the sample, and hence its level of order. At 0.89 Å, the mean B value is 8.3 Å^2 and the width of the distribution is small. In contrast, at 1.96 Å, the mean B is 25.5 Å^2 and the spread correspondingly large. Thus, for the 0.89 Å crystal, most atoms contribute to the high-resolution terms, whereas for the 1.96 Å crystal, only the atoms with lower B values do so. The thin line shows the theoretical inverse gamma distribution $\mathrm{IG}(B) = (b/2)^{d/2}/\Gamma(d/2)B^{-(d+2)/2}\exp[-b(2B)]$, where b and d are the parameters of the distribution, and Γ is the gamma function. For this figure, the values $b = 2$ and $d = 10$ were chosen, which correspond to a mean B value of 20 Å^2 and σ_B of 11 Å^2. In the gamma distribution, the abscissa was multiplied by $8\pi^2$ to make it comparable with the measured B values. All three histograms were normalized to the same scale.

pragmatic approach has been that data extending to 1.2 Å or better with at least 50% of the intensities in the outer shell being higher than 2σ is the acceptable limit (Sheldrick, 1990; Sheldrick & Schneider, 1997). In practice, this means the statistical problem of refinement is overdetermined. For small-molecule structures, accurate amplitude data are normally available to around 0.8 Å, giving an observation-to-parameter ratio of about seven, allowing positional parameters to be determined with an accuracy of around 0.001 Å. This reflects the high degree of order of such crystals, in which the molecules in the lattice are in a closely packed array.

Crystals of macromolecules deviate substantially from this ideal. Firstly, the large unit-cell volume leads to an enormous number of reflections for which the average intensity is weak compared to those for small molecules (see Table 9.1.1.1 in Chapter 9.1). Secondly, the intrinsic disorder of the crystals further reduces the intensities at high Bragg angles and may lead to a resolution cutoff much less than atomic. Thirdly, the large solvent content leads to substantial decay of crystal quality under exposure to the X-ray beam, especially at room temperature. The upper resolution limit of the data affects all stages of a crystallographic analysis, but especially restricts the features of the model that can be independently refined (Table 18.4.1.2). Solutions to the problem of refining macromolecular structures with a paucity of experimental data evolved during the 1970s and 1980s with the use of either constraints or restraints on the stereochemistry, based on that of known small molecules. With constraints, the structure is simplified as a set of rigid chemical units (Diamond, 1971; Herzberg & Sussman, 1983), whereas using restraints, the observation-to-parameter ratio is increased by introduction of prior chemical knowledge of bond lengths and angles (Konnert & Hendrickson, 1980).

As expected, atoms with different ADPs contribute differently to the diffraction intensities, as discussed by Cruickshank (1999). The relative contribution of the different atoms to a given reflection depends on the difference between their ADPs $\{\exp[-(B_1 - B_2)s^2]$ where $s = \sin\theta/\lambda\}$. Clearly, if the average ADP of a molecule is small, then the spread will also be narrow, and most atoms will contribute to diffraction over the whole range of resolution. When the mean ADP is large, then the spread of the ADPs will be wide, and fewer atoms will contribute to the high-resolution intensities (Fig. 18.4.1.2).

Three advances in experimental techniques have combined effectively to overcome these problems for an increasing number of well ordered macromolecular crystals, namely the use of high-intensity synchrotron radiation, efficient two-dimensional detectors and cryogenic freezing (discussed in Parts 8, 7 and 10, respectively). These advances mean that there is no longer a sharp division between small and macromolecular crystallography, but a continuum from small through medium-sized structures, such as cyclodextrins and other supramolecules, to proteins. The inherent disorder in the crystal generally increases with the size of the structure, due in part to the increasing solvent content. However, it is now tractable to refine a significant number of proteins at atomic resolution with a full anisotropic model (Dauter, Lamzin & Wilson, 1997). This work of course benefits tremendously from the experience and algorithms of small-molecule crystallography, but it does pose special problems of its own. The techniques of solving and refining macromolecular structures thus also overlap with those conventionally used for small molecules; a prime example is the use of *SHELXL* (Sheldrick & Schneider, 1997), which was developed for small structures and has now been extended to treat macromolecules.

An alternative and probably better approach to the definition of atomic resolution would be to employ a measure of the information content of the data. There are a variety of definitions of the information in the data about the postulated model (see, for example, O'Hagan, 1994). A suitable one is the Bayesian definition for quadratic information measure:

$$I_Q(p, F) = \mathrm{tr}(A\{\mathrm{var}(p) - E[\mathrm{var}(p, F)]\}), \qquad (18.4.1.2)$$

where I_Q is the quadratic information measure, p is the vector of parameters, F is the experimental data, var(p) is the variance matrix corresponding to prior knowledge, var(p, F) is the variance matrix corresponding to the posterior distribution (which includes prior knowledge and likelihood), E is the expectation, tr is the trace operator (*i.e.* the sum of the diagonal terms of the matrix) and A is the matrix through which the relative importance of different parameters or combinations of parameters is introduced. For

example, if A is the identity matrix, then the information measure is unitary and all parameters are assigned the same weight. If A is the identity matrix for positional parameters and zero for ADPs, then only the information about positional parameters is included. The appropriate choice of A allows the estimation of information on selected key features, such as the active site.

Equation (18.4.1.2) shows how much the experiment reduces the uncertainty in given parameters. Prior knowledge is usually taken to be information about bond lengths, bond angles and other chemical features of the molecule, known before the experiment has been carried out. In the case of an experiment designed to provide information about the ligated protein or mutant, when information about differences between two (or more) separate states is needed, the prior knowledge can be considered instead as knowledge about the native protein.

However, there are problems in applying equation (18.4.1.2). Firstly, careful analysis of the prior knowledge and its variance is essential. The target values used at present, or more properly the distributions for these values, need to be re-evaluated. Another problem concerns the integration required to compute the expectation value (E). Nevertheless, the equation gives some idea about how much information about a postulated model can be extracted from a given experiment.

This alternative definition of atomic resolution assumes that the second term of equation (18.4.1.2) for positional parameters is sufficiently close to zero for most atoms to be resolved from all their neighbours. Defining atomic resolution using this information measure reflects the importance of both the quality and quantity of the data [through the posterior var(p, F)]. In addition, data may come from more than one crystal, in which case the information will be correspondingly increased. There may be additional data from mutant and/or complexed protein crystals, where, again, the information measure will be increased and, moreover, the differences between different states can be analysed. The effect of redundancy of crystal forms is to reduce the limit of data necessary for achieving atomic resolution, which is equivalent to the advantage of noncrystallographic averaging.

18.4.1.1. *Ab initio phasing and atomic resolution*

Ab initio methods of phase calculation normally depend on the assumption of positivity and atomicity of the electron density. Such methods rely largely on the availability of atomic resolution data. In addition, approaches such as solvent flattening and automated map interpretation benefit enormously from such data. The fact that current *ab initio* methods in the absence of heavy atoms are only effective when meaningful data extend beyond 1.2 Å reinforces the idea that this is a reasonable working criterion for atomic resolution.

18.4.2. Data

The quality of the refined model relies finally on that of the available experimental data. Data collection has been covered extensively in Chapter 9.1 and will not be discussed here.

18.4.2.1. *Data quality*

As can be seen from equation (18.4.1.2), the measure of information about all or part of the crystal contents depends strongly on the quality and quantity of the data. Of course, before the experiment is carried out some questions should be answered. Firstly, what is the aim of the experiment? Secondly, what is the cost of the experiment and what are the available resources? With modern techniques, if synchrotron radiation (SR) is used with an efficient detector, the cost of the experiment for different resolutions does not vary greatly (provided that a suitable quality crystal is available). In practice, the apparent increase in cost to attain high-resolution data will generally provide a saving in terms of the time spent by the investigator, since the interpretation of the resulting electron density is much easier and faster. In general, to answer the same question is much easier and cheaper if high-resolution data are available. In addition, high-resolution data mean that answers to some of the questions which may arise during analysis of the experiment will already be addressable. In contrast, low-resolution data not only make it difficult to answer the question currently being asked, but may also necessitate further experiments to address other problems that arise.

While the information content of the data appears to depend quantitatively on the nominal resolution, in fact it is dependent on the data quality throughout the resolution range, and both high- and low-resolution completeness and their statistical significance affect the information content of the data and derived model. High-intensity low-resolution terms remain important for refinement at atomic resolution, as they define the contrast in the density maps between solvent and protein, and because their omission biases the refinement, especially that of parameters such as the ADPs. The rejection of low-intensity observations will have a similar biasing effect. In particular, all the maps calculated for visual or computer inspection by Fourier transformation are diminished in quality by omission of any terms, but are especially affected by omission of strong low-resolution data. This is particularly true in the early stages of structure solution, where low-resolution data can be vital. Although most phase-improvement algorithms rely on relations between all reflections, terms involving low-resolution reflections will be large, will be involved in many relations and will play a dominant role. Hence, omission of these terms will severely degrade the power of these methods, which may indeed converge to solutions that have nothing whatsoever to do with the real structure.

18.4.2.2. *Anisotropic scaling*

The intensity data from a crystal may display anisotropy, *i.e.*, the intensity fall-off with resolution will vary with direction, and may be much higher along one crystal axis than along another. If the structure is to be refined with an isotropic atomic model (either because there are insufficient data or the programs used cannot handle anisotropic parameters), then the fall-off of the calculated F^2 values will, of necessity, also be isotropic. In this situation, an improved agreement between observed and calculated F^2 values can be obtained either by using anisotropic scaling during data reduction to the expected Wilson distribution of intensities, or by including a maximum of six overall anisotropic parameters during refinement. This will result in an isotropic set of F^2 values. For crystals with a high degree of anisotropy in the experimental data, this can lead to a substantial drop of several per cent in R and $R_{\rm free}$ (Sheriff & Hendrickson, 1987; Murshudov *et al.*, 1998).

This ambiguity effectively disappears with use of an anisotropic atomic model. The individual ADPs, including contributions from both static and thermal disorder, take up relative individual displacements, but also the overall anisotropy of the experimental F^2 values. The significance of the overall anisotropy is a point of some contention, and its physical meaning is not clear. It may represent asymmetric crystal imperfection or anisotropic overall displacement of molecules in the lattice related to TLS parameters. Refinement of TLS parameters, which can be performed using, for example, *RESTRAIN* (Driessen *et al.*, 1989), removes the overall crystal contribution to the ADP.

references