# Macromolecular Structure Determination by X-ray Crystallography

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X-ray diffraction is a well-established method to elucidate the atomic structure of singlecrystal macromolecules. An image of the macromolecule forming the crystal cannot be directly recorded as the X-ray phase information is lost during the diffraction experiment. Through systematic variation of the chemical content in the crystal and/or through small changes in the wavelength of the incident X-ray beam, however, a sharp image can be reconstituted computationally. Within the Protein Data Bank, the vast majority of threedimensional structures available have been determined using X-ray diffraction. These structures are used to correlate macromolecular structure with function, to study molecular mechanisms and serve as templates for structure-based drug design of novel therapeutic agents for the treatment of many diseases.

# Introduction

"There is something about protein crystallography that makes it uniquely satisfying. You might work away at a structure, perhaps for years, without an inkling of its nature, until it emerges one day like Venus from the waves and reveals an undreamt of, intricate new facet of nature." Max F. Perutz, March 2000

The analysis of the atomic structure of proteins and nucleic acids is a complex problem and a fascinating area of research in the life sciences. The experimental techniques used for these studies involve single-crystal X-ray diffraction or X-ray fibre diffraction. This article outlines the principles and the key methods involved in macromolecular structure determination by X-ray crystallography.

## History

X-radiation was discovered and applied by Roentgen in 1895. Von Laue and colleagues conducted the first X-ray diffraction experiments using rock salt (Figure 1a) and other alkali halides as crystalline samples. Von Laue's discovery and his mathematical formulation of X-ray diffraction from crystals earned him the Nobel Prize for Physics in 1914. Independently, W. L. and W. H. Bragg carried out similar studies. W. L. Bragg found that the diffraction phenomenon could be treated mathematically as reflection by successive parallel planes passing through crystal lattice points (see Figure 2b). The Braggs, father and son, were both awarded the Nobel Prize for Physics in 1915.

During the 1920s and 1930s the focus of X-ray diffraction studies shifted to more complex systems such as macromolecular fibres and protein crystals. W. T. Astbury and

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doi: 10.1038/npg.els.0002723

coworkers pioneered structural studies on large fibrous proteins such as hair, wool and guills and on DNA fibres. After taking the first fibre diffraction images of DNA, he correctly predicted the overall dimensions of the molecule and found that the nucleotide bases were stacked at intervals of 3.3 Å perpendicular to its long axis. However, it was left to Watson and Crick to elucidate the detailed atomic structure of the DNA double helix. In the Cavendish Laboratories at Cambridge, J. D. Bernal and D. Crowfoot were investigating the diffraction properties of pepsin crystals and recognized that these crystals must be kept in an aqueous, more native-like environment (mother liquor) rather than as dry-mounted crystals. In 1937, Max Perutz performed the first experiments in Cambridge to discover whether it might be possible to determine the structure of haemoglobin by X-ray diffraction. It would take Perutz until 1953 to achieve the most critical breakthrough in actually visualizing the complex molecular structure of haemoglobin. He succeeded in incorporating heavy atoms, namely those of mercury, into definite positions in the haemoglobin crystals (see Perutz, 1992). By this means the diffraction pattern is altered significantly, and the changes can be utilized to determine a direct image of the molecular structure of the haemoglobin. Using the same technique Kendrew succeeded in incorporating heavy atoms (mercury and gold) into myoglobin crystals. This approach provided a solution for an often-insurmountable problem in X-ray structure determination known as the phase problem. By 1958, the structures of myoglobin and haemoglobin had been determined after more than 20 years of dedicated labour, and for these groundbreaking discoveries Perutz and Kendrew were awarded the Nobel Prize in Chemistry in 1962. For their work on the structure of the



**Figure 1** X-ray diffraction patterns. (a) The first diffraction pattern of rocksalt (NACL) recorded in 1911 by Laue and co-workers. (b) A high-resolution diffraction image of a lysozyme crystal recorded with a prototype CCD detector at beamline A1 at MacCHESS, Cornell, USA.

DNA double helix J. D. Watson, F. H. C. Crick and M. H. F. Wilkins received the Nobel Prize in Physiology and Medicine in the same year. Within the next five years the first structures of the enzymes lysozyme, carboxypeptidase, RNase S, chymotrypsin, subtilisin and papain were determined at near atomic resolution.

Diffraction methods are still the most commonly used and successfully applied techniques for elucidating mac-



**Figure 2** The interaction of X-ray photons with matter. Principally, the electric field of the X-ray photons induces in-phase dipole oscillations in the electrons of the two electrons in the sample, which in turn give rise to coherently diffracted radiation. (a) The two electrons in the sample are separated by the distance **r**, the vectors  $\mathbf{s_0}$  and **s** are unit vectors describing the direction of the incident primary beam and the scattered rays, respectively. The path difference gives rise to interference between the scattered beams. (b) Scattering from crystalline lattice. The incident beam approaching the lattice plane at an angle  $\theta$  is reflected from that plane at an equal angle (*Glanzwinkel*).

romolecular structure. The Protein Data Bank (PDB) currently lists more than 20 000 entries with approximately 85% of the deposited structures determined by singlecrystal diffraction. Recent advances in cryogenic techniques at liquid nitrogen or liquid helium temperatures have allowed high-resolution studies using both electron and X-ray crystallography. Major advances in multidimensional nuclear magnetic resonance (NMR) techniques now contribute significantly to our knowledge of the structure and in particular the dynamic properties of macromolecules. Correlating structure (and dynamics) with function provides a more complete understanding of proteins or nucleic acids.

# Single Crystal X-ray Diffraction

Diffraction occurs when X-ray photons interact with electrons (in the biological macromolecule). The electric field of the X-ray photons induces in-phase dipole oscillations in the electrons, which in turn give rise to coherently diffracted radiation. A very simple experiment in which a collimated beam of X-ray photons interacts with two electrons is illustrated in **Figure 2a**. The two electrons are separated by the distance **r**, the vectors  $\mathbf{s}_0$  and **s** are unit vectors describing the direction of the incident primary beam and the scattered rays, respectively. The angle between  $\mathbf{s}_0$  and  $\mathbf{s}$  is typically denoted 2 $\theta$ . The path difference is defined as in eqn [1], where **S** is the scattering vector.

$$\frac{\mathbf{r}\cdot\mathbf{s}}{\lambda} - \frac{\mathbf{r}\cdot\mathbf{s}_0}{\lambda} = \mathbf{r}\cdot\mathbf{S}$$
 [1]

The length of S is a function of the X-ray wavelength and the total scattering angle (eqn [2]).

$$S^{2} = \frac{s^{2} + s_{0}^{2} - 2 \mathbf{s} \cdot \mathbf{s}_{0}}{\lambda^{2}} = \frac{2 \sin^{2} \theta}{\lambda^{2}}$$
$$\times |S| = \frac{2 |\sin \theta|}{\lambda}$$
[2]

In principle, this equation can also be applied to a threedimensional lattice of a single crystal where atoms (and electrons) are ordered in lattice planes (**Figure 2b**). The incident beam approaching the lattice plane at an angle is reflected from that plane at an equal angle (*Glanzwinkel*). Laue discovered that the conditions for observing a maximum in diffracted intensities requires the path difference between reflected beams from adjacent lattice planes be an integral number of wavelengths (h = Sa, k = Sb or l =Sc). With the distance between lattice planes defined as d= 1/|S| and  $|S| = 2|\sin \theta|/\lambda$ , it follows that  $n\lambda = 2d \sin \theta$ . W. H. Bragg first derived this equation in 1912.

Biological matter interacts only weakly with electromagnetic radiation, as the sample is not densely packed with electrons. Proteins and nucleic acid typically consist of atoms of light elements such as hydrogen, carbon, nitrogen, oxygen, phosphorus and sulfur. To enhance the diffraction signal it is necessary to increase the intensity of the incident beam as well as the scattering volume in ordered arrays of macromolecules. This is achieved by shining collimated X-radiation (preferably highly intense synchrotron radiation beams) onto single crystals – highly ordered, symmetrical, three-dimensional arrays. According to Laue and Bragg, scattered intensities arising from crystals result in sharply ordered diffraction maxima (diffraction spots). Symmetry operations such as rotation axes, mirror planes, glide planes, rotation-inversion axes or combined rotation/translation elements are used to describe the geometric relationships between the different molecules forming the crystal lattice. However, certain re-



**Figure 3** This figure shows a primitive, a C-centred, a face-centred and a body-centred orthorhombic unit cell. The orthorhombic system is characterized by orthogonal unit cell vectors of different lengths ( $a \neq b \neq c$  and  $\alpha = \beta = \gamma = 90^{\circ}$ ).

strictions apply in the case of proteins and nucleic acids. They contain chiral centres (L-amino acids, D-riboses, etc.), which in turn are incompatible with the formation of those space groups that contain inversion centres and glide planes. These restrictions limit the number of possible space groups for chiral molecules to 65.

The repeat unit or unit cell is defined by the vectors **a**, **b** and **c** and by the angles between them  $(\alpha, \beta, \gamma)$ . Only seven different types of cells are required to describe a vast array of different packing arrangements and crystal systems: triclinic, monoclinic, orthorhombic, rhombohedral, tetragonal, hexagonal and cubic. These space groups can be further subdivided into 14 distinct Bravais lattices, considering additional symmetry elements on the faces or the inner centre of the unit cells. The most common macromolecular crystal lattices belong to the monoclinic and orthorhombic space groups. The orthorhombic system, for example, is characterized by strictly orthogonal unit cell vectors of different length ( $a \neq b \neq c$  and  $\alpha = \beta = \gamma = 90^{\circ}$ ; see Figure 3). In addition to measuring the unit cell dimensions it is important to determine the space group of the crystal. This is accomplished by analysing the location of the diffraction spots as well as symmetric repeats in diffraction intensities and systematic absences (extinctions). The symmetry of the diffraction pattern accurately corresponds to the symmetry of a given sample space group.

## The Phase Problem

The total observed diffraction  $I_{tot}(S)$  is directly proportional to  $F_{tot}^2(S)$ , the square of the sum of all atomic form factors f(S) and positions x, y, z contained within the asymmetric unit (eqn [3]).

$$F_{\text{tot}}(S) = \sum_{x} \sum_{y} \sum_{y} \sum_{y} f(S)$$
$$\times \exp[2\pi i S(xa + yb + zc)]$$
[3]

Inserting the Laue conditions, S = h/a, S = k/b or S = l/c, eqn [4] follows.

$$F_{\text{tot}}(h,k,l) = \sum_{x} \sum_{y} \sum_{y} f(S)$$
$$\times \exp[2\pi i S(hx + ky + lz)] \qquad [4]$$

The molecular transform  $F_{tot}(h, k, l)$  is a complex quantity consisting of a real and an imaginary part. During a diffraction experiment, only the square of the amplitude  $|F|^2$ , i.e. the real part of an individual Fourier component, can be directly recorded in the form of diffraction spots. However, diffraction intensities are modulated by interference fringes arising from arrays of atoms within the crystal lattice. As seen in Figure 2a and Figure 2b, the modulation is a consequence of phase shifts along the path differences in the lattice. A complete extinction of diffracted intensities occurs when the scattered beams differ by a phase angle of exactly 180°. For example, a translation of the crystal relative to the incident beam or a shift of origin does not cause an apparent change in diffraction intensities or phases. It is not possible to record these phase shifts directly. Therefore, phase information contained in the imaginary part of the molecular transform is lost in the X-ray diffraction experiment and the image (which is dominated by phase information) cannot be reconstructed in a straightforward manner. This experimental dilemma is often referred to as the 'phase problem'. A useful graphical representation of this complex quantity F has been devised in the form of Argand diagrams where individual components  $f_{hkl}$  of the total molecular transform are plotted as vectors in the complex plane with unit vectors A along the real axis (the amplitude |F|) and B along the imaginary axis (the phase angle).

In 1951, Max Perutz succeeded in overcoming this problem for the first time by introducing additional intensity modulations in the diffraction pattern. These particular differences arose from heavy-atom scatterers such as mercury or gold compounds added to pre-grown haemoglobin crystals. The Argand diagrams in **Figure 4a** and **Figure 4b** indicate that the formation of two heavy atom derivatives is sufficient to determine unambiguously the phase (or part *B*) of each Fourier component. The phase problem can be solved only if the lattices of the native crystal and the heavy-atom derivative are isomorphous, i.e. if differences in diffraction intensities are due only to the addition of the heavy-atom scatterers and not a consequence of global (mechanical) changes in the crystal lattice. Recent advances in computational approaches using Bayesian statistics and maximum-likelihood methods have helped to overcome problems in phase refinement and facilitated the accuracy of phase determination (the program SHARP).

Image reconstruction from an X-ray diffraction pattern can also be achieved by other experimental techniques such as single isomorphous replacement in conjunction with anomalous scattering (SIRAS), multiple-wavelength anomalous dispersion (MAD, pioneered by Wayne Hendrickson) or by direct ab initio phasing methods. SIRAS and the MAD method, rapidly becoming the phasing method of choice, are based on the effect of anomalous dispersion. The phenomenon of anomalous dispersion is wavelengthdependent and usually occurs in heavy atoms such as sulfur, bromine, iodine and, prominently, in main and transition group metals. The anomalous effect is caused by the interaction of X-ray photons with outer shell electrons. Some photons may be absorbed and re-emitted at lower energy (fluorescence) but, more importantly, some photons are absorbed in a wavelength-dependent manner and immediately re-emitted at the same energy. Such a scattered photon gains an additional imaginary component to its phase, indicating that it is being retarded compared to a normally scattered photon. Accordingly, the atomic form factors of heavy atoms should be separated into several components (eqn [5]).



**Figure 4** Graphical evaluation of a phase angle. (a) Phase circle or Argand diagrams showing observed amplitudes from the native ( $F_P$ ) and a derivatized form of a protein ( $F_{PH'}$ ) in the complex plane. The phase angle can assume two different values (A or B) and, thus, the phase ambiguity cannot be resolved by a single derivative. (b) With information from two isomorphous derivatives ( $F_{PH'}$  and  $F_{PH''}$ ) it is possible to unambiguously assign a phase value by three intersecting phase circles (B).

$$f_{tot} = f_{norm}(\theta) + f_{anom}(\lambda)$$
  
=  $f_{norm}(\theta) + f'_{anom}(\lambda) + if''_{anom}(\lambda)$  [5]

The differences in intensities (Bijvoet differences) and in phase shifts (retardation) can be utilized to overcome the phase problem. The differences in anomalous amplitudes are usually considerably smaller than those obtained from MIR and therefore MAD experiments require a tunable synchrotron radiation source to optimize the signal and improve accuracy in measuring individual amplitudes by more sensitive detectors. Ab initio phasing methods are based upon the analysis of amplitude triplet and quadruplet inequalities, maximum entropy, or phase averaging requiring prior knowledge about molecular shape and crystal packing. For very large structures with more than 10 000 atoms the phase problem remains a serious obstacle even with novel computational approaches. Macromolecular structure solution from first principles cannot yet be run as a service as in small-molecule crystallography. However, structure determination is increasingly facilitated through molecular replacement, whereby experimental X-ray amplitudes are phased with complementary data derived from an ever growing number of homologous structures, cryo-electronmicroscopic molecular envelopes, and, to a lesser extent, structures determined by NMR.

## Instrumentation

In structural biology laboratories, X-radiation is generated under high vacuum by bombarding a copper or molybdenum target with electrons (accelerated at 50 kV). The deceleration of electrons by the copper anode generates an electromagnetic spectrum consisting of a *Bremsstrahlung* continuum as well as two sharp peaks, which are due to transitions in the discrete electronic energy levels of the copper. Monochromatic radiation is obtained by passing the *Bremsstrahlung* through a nickel filter and a system of platinum-coated mirrors.

In recent years, synchrotrons have had a major impact on the analysis of macromolecular structure. Synchrotron rings (and other storage rings) are large devices in which electrically charged particles circulate at close to the speed of light. The charged particles generate electromagnetic radiation when they are diverted from a straight path by a magnetic field generated by a bending magnet. Inserted devices such as multipole wiggler magnets or undulators further modify the path of the electrons (or positrons) and allow modification of the X-ray wavelength. The wavelength can now be selected to reduce radiation damage or tuned to meet specific attributes of the biological sample or bound ligands. This has been particularly successful in utilizing anomalous diffraction characteristics (or X-ray fluorescence) of heavier chemical elements such as Se, Fe, Zn, Cu, etc.

Although protein or nucleic acid crystals are usually only fractions of a millimetre in size, they contain  $10^{12}-10^{14}$ crystallographically ordered molecules. The diffraction pattern arising from such tiny crystals is imaged by proportional counters, X-ray sensitive films or imaging plates or, more directly, by electronic readout devices (chargecoupled device, CCD). Installation of electronic data collection devices resulted in a dramatic increase in data acquisition and processing speeds by orders of magnitude, reducing the experimentation time from days to minutes.

# **Crystallization and Structure Determination**

Structure determination is a complicated, involved process consisting of several crucial steps with potential shortfalls that must be overcome. The rate-limiting step in macromolecular crystallographic studies is the production of single crystals. Crystallization is achieved by slowly bringing the sample from a state of supersaturation to the crystalline state, avoiding nonspecific aggregation. This is achieved by a variety of methods such as vapour diffusion, (micro-)batch crystallization, (micro-)dialysis or free interface diffusion. The crystallization process is affected by sample saturation, concentration of precipitants and ionic strength, buffer concentration and pH, temperature, and the use of detergents and other organic additives. It is crucial to approach the nucleation point slowly and reproducibly. In some cases, seeding with previously grown crystals or micro-crystalline material will trigger nucleation and crystal growth of new sample preparations.

Initially, crystallization trials are carried out over a large variety of different conditions, for which millimolar amounts of sample are required. Such quantities are often only accessible through recombinant techniques (exceptions are highly abundant macromolecules such as haemoglobin, porins, or large assemblies such as ribosomes and proteosomes). Much smaller sample volumes are required and crystallization trials with nanolitre quantities are being carried out with the help of robotic systems. Small oligopeptides of fewer than 200 amino acids can be synthesized but require refolding to a native state. Nucleic acid molecules of fewer than 100 nucleotides can be chemically synthesized, but larger RNA samples have to be generated by *in vitro* transcription from appropriate DNA templates by using T7 RNA polymerase.

Once experimental phases have been obtained through MIR and MAD or through direct calculation from a homologous atomic model (molecular replacement), the electron density equation can be solved and an image for the molecular transform can be completed using eqn [6], where V is the volume of the asymmetric unit cell and N is the number of molecules contained within this volume.

$$\rho(x, y, z) = \frac{1}{NV} \sum_{-h}^{h} \sum_{-k}^{k} \sum_{-l}^{l} f(S) \exp(-i\alpha)$$
$$\times \exp[-2\pi i(hx + ky + lz)]$$

$$\exp\left[-2\pi i(hx+ky+lz)\right]$$

[6]

The electron density is usually only a rough outline of the crystallized sample and requires further interpretation using graphical and computational means. Major advances in molecular graphics software (e.g. the programs O, Quanta, MAIN and others) such as interactive modelling, fragment fitting, online consultation of structure databases, on-the-fly contouring and calculation of difference electron density maps have greatly facilitated the manual interpretation and intervention in building an initial structural model. The initial model is then subjected to further computational optimization (structure refinement), which is required to produce a plausible, geometrically sound macromolecular model.

Structure refinement uses geometrical constraints such as proper bond lengths, bond and tetrahedral angles, planarity, backbone angles as guides to optimize the model structure against the experimental data. Refinement programs such as CNSsolve, REFMAC and TNT attempt to minimize the difference electron density between the experimental and model maps, either by maximum-likelihood or by the least-squares methods.

# Milestones

The explosion in the number of crystal structures deposited with the Protein Data Bank is a compelling testimony for the power of X-ray crystallography. Here, four examples have been selected to illustrate the wealth of information brought forward by high-resolution structural studies.

#### Haemoglobin and myoglobin

Myoglobin and haemoglobin, the first proteins for which full three-dimensional structures were determined at high resolution, play a crucial role in oxygen transport and storage in the muscle. Like other members in this family of proteins, they consist entirely of  $\alpha$ -helices. In myoglobin, there are a total of eight helices of which two, E and F, are oriented such that they form a V-shaped pocket. In the holoenzyme the pocket contains the haem group (prosthetic group or cofactor), a large heterocyclic ring containing four pyrrole rings. The centre of haem is occupied by an  $Fe^{2+}$  cation (Figure 5).

The histidine residue adjacent to the iron (proximal - on the left) is important in mediating the noncooperative binding of oxygen to the protein. In haemoglobin, a similar arrangement of residues can be found in the haem binding pocket. However, oxygen binding and release is allosterically regulated by the pseudo-tetrameric arrangement (a dimer of dimers;  $\alpha_2\beta_2$ ) of the individual protein subunits.



(b)

Figure 5 Myoglobin (a) and haemoglobin (b), the first proteins for which full three-dimensional structures were determined at high resolution. (a) Note the electron density corresponding to the haem cofactor in the Vshaped binding pocket. The map is contoured 2.5 Å above the mean electron density. (b) The heterodimer of haemoglobin. Like other members of this family of proteins, it consists entirely of a helices (secondary structure). Both proteins play a crucial role in oxygen storage and transport in the muscle.

#### **Reaction centres**

Photosynthetic reaction centres (RC) are crucial catalysts in the photosynthetic process, perhaps the most important chemical reaction in the biosphere. The conversion of light to chemical energy is a prerequisite for all higher life on Earth. RCs are large multiprotein complexes located in the outer membranes of plants and bacteria. The X-ray struc-



**Figure 6** Photosynthetic reaction centres (RC) are crucial catalysts in the photosynthetic process, perhaps the most important chemical reaction in the biosphere. The conversion of light to chemical energy is a prerequisite for all higher life on earth. RCs are large multiprotein complexes located in the outer membranes of plants and bacteria. The X-ray structure of the reaction centre is the first structure of an integral membrane protein determined at high resolution. There are four protein chains: H (yellow), L (blue) and M (green) subunits, and cytochrome (red).

ture of the reaction centre is the first structure of an integral membrane protein determined at high resolution. There are four protein chains: the H, L and M subunits, and cytochrome c (Figure 6). The H chain has one transmembrane helix, while the L and M chains have five each. The cytochrome c subunit has no membrane-spanning helix, it is anchored by proteins L and M. The crystal structure shows how the photosynthetically active components bacteriochlorophyll, bacteriopheophytin, quinone and the haem groups are arranged. The spatial arrangement of these chromophores reveals the path and the order of the previously postulated electron transfer steps.

#### **Bluetongue virus**

Bluetongue virus (BTV) belongs to the family of Orbiviruses. The structure of the core of BTV has a diameter of 700 Å and represents the largest particle yet solved by X-ray crystallography. The structure illustrates in atomic detail how nearly 1000 protein subunits self-assemble and interact to form a transcriptionally active compartment. Interestingly, the structure also reveals how double-stranded RNA is packaged in the interior of the core particle (Figure 7). Addition of magnesium ions and nucleotide triphosphates activates the replication machinery contained within the core.



**Figure 7** The structure of the Bluetongue virus (BTV) core has a diameter of 700 Å and represents the largest particle to date solved by X-ray crystallography. The structure illustrates in atomic detail how nearly 1000 protein subunits self-assemble and interact to form a transcriptionally active compartment.



**Figure 8** The structure of the 50S ribosomal subunit has been determined and refined at 2.4 Å resolution, while the 30S particle has been determined to 2.8 Å. The high-resolution structures of the particle show in detail the binding sites for the amino-acylated tRNAs and for elongation factors, and a long tunnel that is used as an exit by the emerging polypeptide chain. The structure of the 50S subunit also reveals that large portions of the subunit are built up from RNA. In fact, the active site entirely consists of RNA, implying that the ribosome is a ribozyme.

## **Ribosomal subunit**

Ribosomes are large molecular assemblies (cytoplasmic organelles) consisting of complexes of proteins and in eukaryotes up to four large RNA molecules. A large (50S) and a small (30S) subunit are loaded onto an mRNA molecule to mediate the translation of the genetic message into

a specific sequence of amino acids, or a polypeptide chain. The structure of the 50S subunit has now been determined and refined at 2.4 Å resolution, while the 30S particle has been determined to 3.0 Å (Figure 8). The high-resolution structure of the 50S particle shows in detail the binding sites for the aminoacylated tRNAs and for elongation factors, and a long tunnel that is used as an exit by the emerging polypeptide chain. The structure also reveals that large portions of the 50S subunit are built up from RNA. The ribosomal proteins play only a subordinate, architectural role and do not directly participate in the peptidyltransferase activity of the ribosome. X-ray crystallography provides direct proof that the ribosome is in fact a ribozyme (catalytic RNA).

# **Further Reading**

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