Review

After the Revolution: Where X-Ray Crystallography Stands in Context with Cryo-Electron Microscopy

Après la Révolution : où la cristallographie aux rayons X se situe dans le contexte de la cryomicroscopie électronique

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Abstract | Résumé

Despite the surge of cryo-electron microscopy (cryo-EM) as a leading method for structural determination in recent years after its "resolution revolution," X-ray crystallography continues to play a key and crucial role in the field of structural biology. Although cryo-EM makes it possible to resolve non-crystallizable macromolecules, X-ray crystallography remains more effective in achieving atomic resolution and has the ability to define side chain and ligand orientations and interactions, making it useful in the mechanistic and enzymological study of proteins. This review highlights X-ray crystallography and emphasizes that X-ray crystallography and cryo-EM remain complementary methods in determining macromolecular structures instead of the former being fully replaced by the latter. In this context, together with other methods for structural analysis, X-ray crystallography is shown to adapt to the evolving structural biology landscape.

Malgré l'essor de la cryomicroscopie électronique (cryo-ME) comme méthode de premier plan pour la détermination des structures ces dernières années après sa « révolution de la résolution », la cristallographie aux rayons X continue de jouer un rôle clé et crucial dans le domaine de la biologie structurale. Bien que la cryo-ME permette de résoudre des macromolécules non cristallisables, la cristallographie aux rayons X reste plus efficace pour atteindre la résolution atomique et a la capacité de définir les orientations et interactions des chaînes latérales et des ligands, ce qui la rend utile dans l'étude mécanistique et enzymologique des protéines. Cette revue met en lumière la cristallographie aux rayons X et souligne que la cristallographie aux rayons X et la cryo-ME restent des méthodes complémentaires pour déterminer les structures macromoléculaires, au lieu que la première soit entièrement remplacée par la seconde. Dans ce contexte, avec d'autres méthodes d'analyse structurale, la cristallographie aux rayons X s'est révélée s'adapter au paysage évolutif de la biologie structurale.

Keywords: X-ray crystallography; Cryo-electron microscopy; Cryo-EM; Structural biology; Crystallization; Resolution; Resolution revolution

Introduction

Until the twentieth century, much of the molecular world was invisible because it could not be seen with the light microscope (1). With the emergence of the field of structural biology, however, the structure, function, and behaviour of biological macromolecules, particularly proteins, could be predicted based on physical properties and sequences (2). New protein structures continue to be determined routinely, enhancing the understanding of molecular processes and contributing to biotechnological and medical breakthroughs (1, 3). This was made possible through imaging techniques such as X-ray crystallography, cryo-electron microscopy (cryo-EM), or nuclear magnetic resonance spectroscopy.

As of today, X-ray crystallography is the most productive technique, with over 200,000 total contributed structures in the Protein Data Bank (PDB), relative to cryo-EM, which contributed only 33,000 structures (4, 5). However, recent improvements in cryo-EM-related software and hardware have allowed the technique to go through a "resolution revolution," surpassing X-ray crystallography in the determination of certain types of protein structures, such as large or membrane proteins (5, 6). As a whole, the determination of cryo-EM structures also continues to increase exponentially, with over 7,000 cryo-EM submissions to the PDB compared to 10,000 X-ray crystallography submissions in 2025 (7, 8). The field of structural biology is thus changing dynamically, with some arguing that X-ray crystallography's relevance is becoming insignificant (5). This review contests this claim, demonstrating how X-ray crystallography not only remains a key technique within the field, but also has the ability to adapt to and complement the changing landscape.

X-Ray Crystallography

Preceding electron microscopy techniques, X-ray crystallography was invented in 1912 and continues to be used to produce most of the protein structures deposited in the PDB. The technique uses X-rays and takes advantage of their having wavelengths that match the interatomic distance of crystals (5). X-rays can thus be diffracted by crystals and resultantly depict the crystal's arrangement of atoms. As such, X-ray crystallography requires crystallized protein and a diffraction pattern to build a structural model (9).

Protein crystallization

X-ray crystallography requires the formation of crystals (i.e., a well-ordered 3D packing of homogenous molecules), for structural determination. This is the bottleneck of the process, with many proteins failing to be resolved using crystallography due to their inability to crystallize. The principle of crystallization is to induce the protein to slowly precipitate out of a supersaturated solution (10). In a supersaturated solution, the amount of protein present exceeds its solubility limit, so protein is pushed out of solution (11). However, if this happens too quickly, then amorphous precipitation occurs instead. Other factors, such as the protein concentration, buffer contents, pH, temperature, and crystallization method also affect the crystallization process, with initial experiments requiring significant trial and error to establish the optimal set of conditions to encourage crystallization for a given macromolecule (10).

The most frequently used method to achieve supersaturation and crystallization of a protein is vapor diffusion. This method includes two different techniques: hanging-drop vapor diffusion and sitting-drop vapor diffusion. In both techniques, a drop of protein solution is mixed with an equal volume of precipitating solution, with the drop/mixture either hanging over a reservoir solution for hanging-drop (11) or, if the mixture has low surface tension (12), seated above the reservoir solution on a platform in sitting-drop. The concentration difference between the drop and the reservoir solution drives the system towards equilibrium via water vapor diffusion from the drop to the reservoir, thus concentrating the protein and promoting crystallization (11).

Diffraction data collection and analysis

In X-ray crystallography, a monochromatic X-ray beam collides with the crystal that is rotated, and a transducer behind the sample detects and counts the number of photons that collide into it, displaying spots of different intensities and arranged in a particular pattern. This is the diffraction pattern of the crystal of interest, and it is specifically related to the crystal morphology and protein structure. Diffraction occurs when an X-ray encounters an atom's electron cloud (instead of passing through the crystal) and bends around said atom (12, 13).

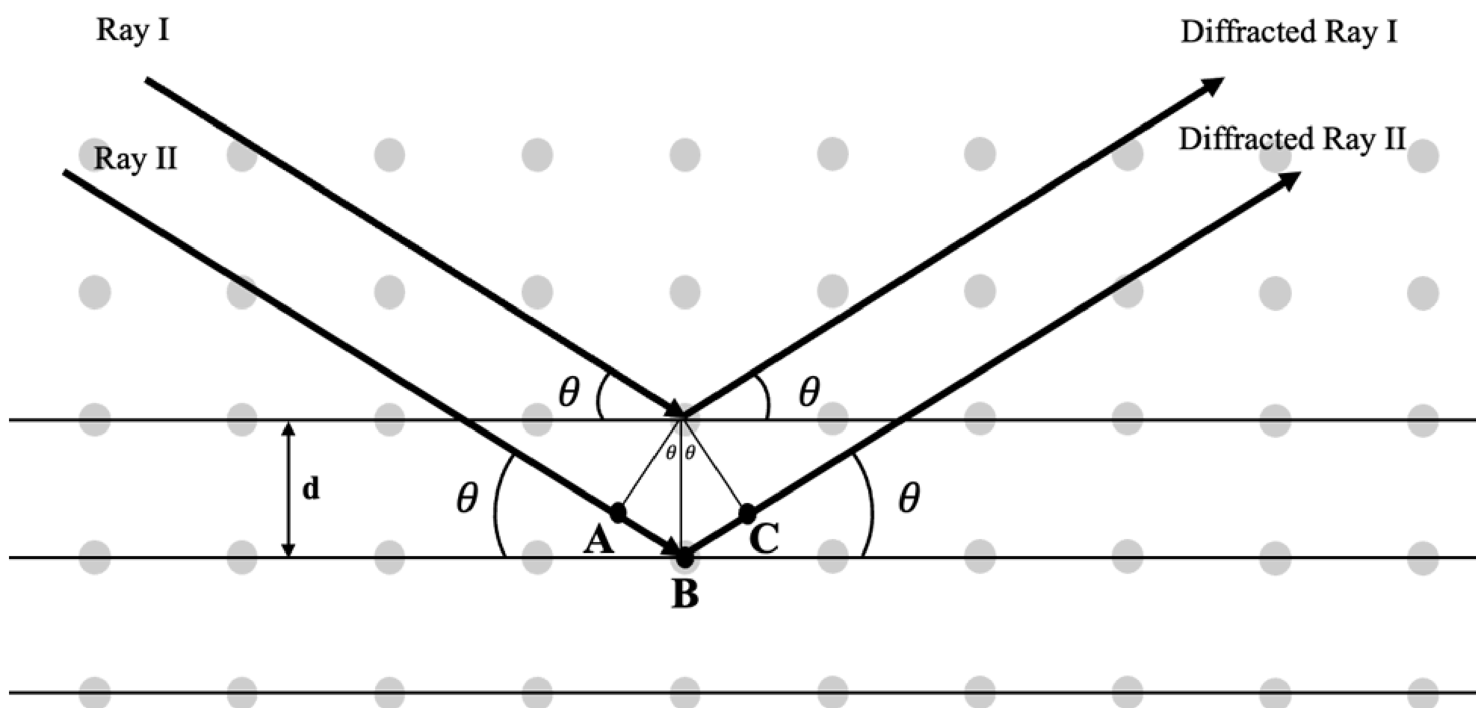

Figure 1. Bragg's Law. In a crystal lattice, atoms (grey circles) are regularly distanced, forming parallel equidistant (distance d) planes. Ray I and Ray II will diffract from identical atoms in adjacent unit cells and interfere. For constructive interference that results in a diffraction spot, Ray II must diffract in phase with Ray I. This will only occur if the extra pathlength traveled by Ray II (i.e., AB + BC) is equivalent to an integer of the wavelength of Ray I. Geometrically, this is equivalent to $2d \sin\theta = n\lambda$, which is known as Bragg's Law.

The resulting diffracted waves can also interfere constructively or destructively. In the former, two waves interact in phase and combine to form a wave of larger amplitude, while in the latter,
two waves interact out of phase and cancel each other out to form a wave of zero or lower amplitude. Interestingly, because of the repeating ordered nature of atoms within a crystal, an X-ray interacts with equivalent atoms in adjacent unit cells (repeating structural units), on equidistant parallel planes (Figure 1). This means that diffracted rays from adjacent planes will interact with each other (14).

Bragg's Law predicts whether these diffracted rays will interfere constructively or destructively. Since the rays on parallel planes are diffracted at the same angle that they initially interact with the crystal's atom, successive rays will travel different pathlengths before interacting. For example, in Figure 1, diffracted Ray II travels a pathlength of AB + BC more than diffracted Ray I. Bragg's Law states that these diffracted rays will only interact constructively if the difference in their pathlength is equal to an integer of their wavelength (14):

$$AB + BC = n\lambda \quad \text{(Eq. 1)}$$

Where n represents an integer value, and λ the wavelength of the X-ray. Geometrically, this is equivalent to:

$$AB + BC = d\sin\theta \quad \text{(Eq. 2)}$$

Where d is the interplanar distance between the atoms of interaction, and θ the angle of incidence (which is the same as the angle of diffraction).
These two equations can therefore be combined to give the following equation, commonly referred to as Bragg's Law (14):

$$2d\sin\theta = n\lambda \quad \text{(Eq. 3)}$$

Where $2d\sin\theta$ is the total extra pathlength traveled by the diffracted ray (Ray II), and its equivalence to $n\lambda$ being the condition for constructive interference (with Ray I).

Additionally, if X-rays diffracted in a given direction from two identical atoms in adjacent unit cells interfere constructively, then all X-rays diffracted in that direction from identical atoms in adjacent unit cells will interfere constructively, too. The diffraction pattern's spot positions are thus characteristic of the ordered arrangement of atoms within a crystal, and they can be used to determine the unit cell's type, size, and dimensions. Each diffracted X-ray is also a manifestation of a structure factor, which describes the wavelength, intensity, and phase of the combined diffracted ray from a given angle, from every atom in a 3D reciprocal lattice (13). If all structure factor values are known, then the data can be Fourier transformed into a 3D electron density map (13, 14).

However, the phase of the diffracted rays cannot be directly obtained from the diffraction dataset (9). This is known as the "phase problem," but it can be solved indirectly. The two most common methods for this are isomorphous replacement and molecular replacement (10).

In molecular replacement, the structure factors from a closely related protein structure (homologous amino acid sequence) are used and their phases are applied to the protein of interest's dataset to calculate the new structure factor (10). This method is based on the observation that proteins with homologous amino acid sequences undergo very similar polypeptide chain folding and therefore have similar 3D protein structures. Interestingly, molecular replacement can also be used to determine relative positions of subunits or protein molecules within structural complexes (12). Nevertheless, although the reference structure must be placed in the unit cell in the same orientation and position as the protein of interest, there will always be bias towards the existing structure factor calculations (10).

On the other hand, isomorphous replacement is used when no closely related structure is available. Instead, after collecting the initial diffraction dataset, the crystal of interest is soaked in a heavy atom salt solution (e.g., mercury, platinum, gold) to incorporate heavy atoms into the protein molecule without changing its confirmation or the crystal's unit cell dimensions. By comparing the changes in diffraction intensities between this altered crystal's dataset and the original crystal of interest's dataset, the locations of the heavy atoms can be determined and phases can be estimated (10). Notably, while perfect isomorphisms rarely occur, a change in cell dimensions of ¼ of the resolution limit tend to be tolerated (12).

Building a structural model

Finally, the collected X-ray diffraction data and structure factor coordinates are converted into an electron density map to visualise real space atomic positions of the protein via inverted Fourier transformation. Fourier transformation is a mathematical operation that can decompose a signal or function in the form of signal intensities and phases (usually in its frequencies), converting complex data into its more basic components and making the data more understandable (15). The resulting map therefore depicts the 3D contours into which the protein sequence will be fitted, and the structure will be built and refined (10).

Advantages and considerations of X-ray crystallography

With highly developed robust experimental and computational methods, X-ray crystallography can solve protein structures at atomic-level resolution, excelling especially with small- to medium-sized macromolecules. Individual atoms and ligand-receptor interactions can also be resolved within crystallographic structures, allowing X-ray crystallography to be used to deduce exact atomic positions, precise mechanisms of enzymes, the binding of a drug to its target, and the structure of small and stable proteins (16).

However, X-ray crystallography is limited by its need to have highly purified samples of crystallizable protein. Not only does this mean that (should crystallization occur) more flexible proteins, like membrane proteins, may result in a less-resolved structures, but it also means that the protein in a crystal lattice may not be captured in its native-state (16, 17). Similarly, although X-ray crystallography and cryo-EM both provide snapshots of a protein's structure, because crystal structures have lattice-constrained orientations, X-ray crystallography may provide less structures for a given protein compared to cryo-EM (16).

Cryo-EM

General concepts and methodology

Although cryo-EM, primarily developed in the 1980s (5), can refer to different related techniques, the type that is attributed with revolutionizing structural biology is single particle cryo-EM, or single-particle analysis (18). This is a type of transmission electron microscopy, where the interactions of scattered electrons are used to generate contrast in an image. For purified proteins, samples are applied onto a carbon-film-covered copper grid and either embedded in a heavy-element stain (i.e., negative-stained) or flash frozen (i.e., vitrified) for regular transmission electron microscopy or cryo-EM imaging, respectively. By imaging samples dispersed on the thin and continuous carbon film, negative staining assesses the sample's homogeneity and purity and estimates optimal concentrations by revealing the protein samples in good contrast. This, however, introduces additional background noise and reduces the information that can be obtained of the internal protein structure, so it is not a process suitable for high-resolution structural determination. On the other hand, vitrified samples are prepared by depositing protein preparations on a thin and holey carbon film and quickly plunging the sample into a cryogen, like liquid ethane. This avoids the formation of ice crystals that can block imaging of single-particle samples. The protein samples are also frozen within the thin-layered ice in the individual holes on the carbon film, which, in principle, reserves the proteins' native architecture. Additionally, due to the sensitivity of biological specimens, low-dose electron beams are necessary for cryo-EM to image the sample while minimizing radiation damage (18–21).

The electron microscopy data is then collected as micrographs (i.e., a series of two-dimensional (2D) projections of the sample), which, in theory, show images of the sample in different orientations. Particles that show identical views of the same projection are then aligned two-dimensionally and averaged into a "2D classification" to reduce noise, providing a single micrograph of the protein at a given orientation. These signal-enhanced 2D images are then processed in their reciprocal space by Fourier transformation and mathematically combined and convoluted by three-dimensional (3D) image reconstruction. The 3D picture is then plotted as an electron density map into which the protein's amino acid residues can be fitted to generate an atomic model (19, 21).

Advantages and considerations of cryo-EM

Cryo-EM can consistently be used to obtain structures at intermediate to high resolution, without the need for crystals. Additionally, because macromolecules remain in their soluble state before vitrification, this technique can be used to solve structures of proteins with post-translational modifications or flexible domains in more near-native states (9, 22). Although it is true that flexible regions may still be unresolved in a cryo-EM structure, the entire protein can still be used for data acquisition and structure determination. On the other hand, in X-ray crystallography, protein truncation to remove flexible residues is common since it can reduce conformational heterogeneity and enhance lattice formation (11). Similarly, cryo-EM is particularly useful for resolving the structure of membrane proteins, which are more difficult to resolve using crystallography due to their flexibility, instability after extraction from the membrane, and difficulty in purifying the large quantities needed for crystallization (18, 23). Most notably, recent developments within the field now allow cryo-EM to be used for resolving smaller macromolecular structures as well, instead of only being limited to larger structures or supra-assemblies (24, 25). As such, cryo-EM offers great potential in structural determination, and as analysis software continues to improve, it is not surprising that the technique is becoming increasingly popular and possibly preferred.

However, limitations still exist. Because of the thermal motion of proteins before vitrification, heterogeneity in a cryo-EM sample is unavoidable (9), potentially causing regions of a cryo-EM map or structure to have different resolutions or be biased in favour of predominant structure states/conformations over rarer, intermediate ones (26). The sample is also always exposed to radiation damage. High-dose electrons destroy the sample before sufficient images can be obtained for high-resolution structural determination, while lose-dose electrons enhance background noise and reduce resolution. In either situation, the radiation limits the resolution that can be achieved within structures (19). Similarly, grid quality and sample uniformity also remain limiting factors in the quality of cryo-EM-resolved structures, often requiring significant time to achieve optimal conditions for a well-resolved structure. While trial-and-error is still present in crystallization experiments, X-ray crystallography is more well-established, with some facilities offering increased automation for crystal selection. This makes it possible to simultaneously screen multiple crystallization conditions to select for the best diffracting crystal (27).

Furthermore, cryo-EM instrumentation (e.g., electron microscopes and grid plunge-freezing devices) are also expensive, often costing millions of dollars, and requiring high operating and maintenance costs along with specific conditions for optimal performance. This contributes to the instrumentation being less accessible for use by non-expert scientists. Computational requirements are also significant; large datasets must be handled, and many computational resources are required to process the cryo-EM data. Resultantly, labs often need to invest in additional graphics processing unit workstations and storage space (27, 28).

X-Ray Crystallography's Role in a Cryo-EM-Dominant Period

Although X-ray crystallography was established earlier than cryo-EM, recent technological advancements have contributed to remarkable success for single-particle cryo-EM as a leading method for macromolecular structure determination. With cryo-EM no longer limited to resolving structures larger than 200 kilodaltons (24, 25) and achieving near-atomic resolutions, the continued relevance of X-ray crystallography is called into question.

However, to say that the future of structural biology will only be X-ray crystallography or cryo-EM, with the other becoming obsolete, is an overstatement. The future use of either technique not only depends on their own advantages and limitations, but also the biological questions that need to be answered and the goals that must be achieved. Notably, cryo-EM can skip the crystallization bottleneck entirely, offer high resolution for larger, more flexible, and/or disordered macromolecules, and it can provide information on more macromolecule conformations in a single experiment. In contrast, crystallography yields better resolution for macromolecules smaller than a few hundred kilodaltons, and it can provide dynamic information as a function of time, temperature, and pressure, even highlighting protein-ligand interactions and side chain orientations. Most importantly in pharmaceutical discovery, it allows high-throughput screening of drug candidates. X-ray crystallography therefore still plays an essential role in structural biology, as the field's overarching goal is to relate structure to biological function (5).

How X-ray and electron beams interact with samples is also to be considered. X-rays interact with an atom's electrons, so a structure solved by X-ray crystallography reflects the electron cloud distribution (i.e., the electron density) within the macromolecule. Electron beams instead interact with the Coulomb/electric potential of atoms, with electrons being diffracted based on the charge distribution within the sample (9, 29). These two resultant densities thereby represent different physical properties of a macromolecule depending on the resolution quality. At resolutions less than two angstroms, electron and Coulomb potential densities give very similar molecular shapes and features, but this is no longer the case when the resolution is higher than one angstrom. In such a case, the densities will reflect different physical properties of the macromolecule and result in different structures (9). As such, for the time being, cryo-EM cannot simply replace X-ray crystallography. X-ray crystallography is however being repositioned within the field of structural biology, especially in the context of how the two techniques may be used to complement one another to improve the understanding of biological mechanisms.

Complementarity Between X-Ray Crystallography and Cryo-EM

X-ray crystallography and cryo-EM data can complement each other in the determination of accurate structures in many ways. Some method examples include I) docking crystallographic structures within cryo-EM maps, II) solving phases of crystallographic data using cryo-EM maps, and III) crystallizing domains or subunits to fill in non-resolved sections from cryo-EM maps (9).

I) Docking crystallographic structures within cryo-EM maps

There are two key considerations that birthed this complementary technique. The first is that due to the static conformations that are required for crystalline structures, X-ray crystallography does not always reveal interaction modes or full mechanistic insights for a complex. The second is that cryo-EM maps tend to be less resolved than electron density maps from X-ray crystallography. As a result, by docking a higher-resolution crystal structure into a lower-resolution cryo-EM map, the crystal structure can be used as a template to solve the cryo-EM structure at a higher resolution than possible from using the cryo-EM map alone. With the possibility of rigid-body fitting, which aligns domains as fixed units to find optimal positions and orientations, or flexible fitting, which allows the crystal structure to undergo conformational changes (while maintaining stereo-chemistry) to fit the cryo-EM map more accurately, conformational differences between the crystallographic and cryo-EM structures can be identified, unlocking mechanistic insights that would not have been identified from crystallography or cryo-EM, in isolation (9, 30).

II) Solving phases of crystallographic data using cryo-EM maps

In X-ray crystallography, the phase problem largely occurs because there are no lenses that can collect and focus X-rays. On the other hand, electron microscopes like cryo-EM use electromagnetic lenses to collect and recombine scattered waves. As such, both the amplitude and phase information are retained within cryo-EM structure factors, thereby avoiding the same phase problem seen in X-ray crystallography (31). With cryo-EM reconstructions also increasing in resolution with the improvement of processing software, there can now be sufficient resolution overlap between X-ray crystallographic and cryo-EM data (for the same macromolecule) to use the cryo-EM map as an initial phasing model in molecular replacement for determining the crystallographic phases. Additionally, with cryo-EM now able to be used for smaller-sized macromolecules, even at low resolution this method is more applicable and may be used to solve the crystallographic phases in smaller molecules than what was previously possible (9). This method is thus particularly useful to solve the phase problem when no other existing structure exists for molecular replacement, or when isomorphous replacement becomes challenging.

III) Crystallized domains/subunits to fill non-resolved cryo-EM map sections

Although cryo-EM does not require crystallization and can resolve larger macromolecules/complexes, due to the flexible nature of a protein or its domains and the inevitable conformational heterogeneity within a sample, cryo-EM reconstructions may not have evenly distributed resolutions at each point in the density

map. This commonly results in the peripheral structure of the protein being less resolved. However, crystallization of either the entire protein or of individual domains or subunits locks the structure in one rigid conformation, so these lower- or non-resolved regions from a cryo-EM reconstitution can be solved using X-ray crystallography, and its atomic model can be fitted back into the cryo-EM map to improve resolution in these lacking areas. Because samples may also be prepared under different conditions in crystallization versus cryo-EM, the individually solved structures may also represent distinct biological states, which can contribute to a more complete structural representation of the macromolecule (9).

Conclusion

Currently, X-ray crystallography and cryo-EM are both at the forefront of structural biology. Despite recent increased favour for cryo-EM analyses, X-ray crystallography remains a fundamental technique. The distinct advantages for crystallography thus remain relevant, with it being unlikely that cryo-EM may fully replace X-ray crystallography. One method does not need to diminish the other's capabilities, nor be used entirely alone. Instead, the current era of structural biology and increased reach towards cryo-EM is reshaping the role of X-ray crystallography within a complementary framework, where better structures and more complete mechanistic profiles can be obtained in combination than when either technique is used alone. All in all, in structural biology, it is not only a question of resolving a protein structure, but also a question of how the structure elucidates function. Structural biologists must therefore use the method that will most effectively answer the biological question at hand, regardless of a technique's changing relative popularity.

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