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19.2. Electron diffraction of protein crystals

BY W. CHIU

19.2.1. Electron scattering

When an electron interacts with a free atom, it is simultaneously attracted to the nucleus because of the nuclear positive charge and repelled by the electrons of the atom. An electron scattering event is a composite of these forces (Hirsch *et al.*, 1977). Mathematically speaking, an electron 'sees' the potential function of the atom, which can be approximated as a 'screened Coulomb potential function'. This function is often referred to as a mass-density function and is analogous to the electron-density function in the case of an X-ray photon, which is scattered only by the electrons of an atom.

Because of the strong interactions between an electron and an atom, the scattering cross section of an atom is much higher for electrons than it is for X-rays. For a 100 keV electron, it is about 10^4 times greater than for an X-ray. For every single electron scattering event of a carbon atom, there is more than a 60% probability that the electron will lose part of its energy, which is called inelastic scattering. The energy lost is primarily in the range 10 to 20 eV, which is sufficient to induce excitation and ionization of the atoms upon irradiation (Isaacson, 1977). This energy transfer to a molecule results in breakage of chemical bonds and mass migration of broken molecular fragments.

19.2.2. The electron microscope

An electron microscope is conceptually analogous to a light microscope. It consists of an electron source, condenser lenses, an objective lens, projector lenses and a camera recorder. Because of the electronegative property of the electron, it is possible to fabricate magnetic and electrostatic lenses to focus electrons to near atomic resolution. The most critical lens in an electron microscope is the objective lens, which forms the first diffraction pattern at its focal plane and the first image at its image plane. An electron diffraction pattern is the same as an X-ray diffraction pattern, containing only the amplitude information of the structure factor, whereas an electron image contains both the amplitude and phase information of the structure factor (Unwin & Henderson, 1975).

The condenser lens is used primarily to control the beam diameter and the flux of the electrons irradiating the specimen, whereas the projector lenses are used to magnify either a diffraction pattern or an image in a broad range. The camera length in an electron microscope is adjustable, ranging from 0.2 to 2.5 m, and allows the recording of diffraction patterns with Bragg spacings from hundreds to a fraction of an ångstrom. The magnification of an image spans from a hundred to a million times. Magnification is not a limiting factor for image resolution, however, and is typically set between 40 and 80000 times for protein electron crystallography. The most important factors that affect the instrumental resolution are the coherence of the incident electron beam, the chromatic and spherical aberrations of the objective lens, the electrical stability of the electron gun and the objective lens, and the mechanical stability of the specimen stage (Chiu & Glaeser, 1977). In general, almost all modern electron microscopes are capable of resolving a lattice spacing of 2.4 Å in a thin gold crystal. The biological structure resolution in an image of a protein crystal has not reached the instrumental resolution because of many factors related to radiation damage of the specimen. Improvements in experimental and computational methods, however, have made it feasible to image protein crystals beyond 3.7 Å resolution (see below).

The range of electron energy used in an electron microscope is between 20 and 1000 keV, which corresponds to wavelengths of 0.086–0.0087 Å. Any single microscope is built and optimized for a narrow energy range because of the complex design of a highly stable electron gun. The instrument most commonly used for molecular-biology structure research operates in the range 100 to 400 keV. Choosing the most useful electron energy is based on the desired resolution and the specimen thickness. The thicker the specimen, the higher the energy that should be used in order to avoid dynamical scattering effects and to have a sufficient depth of field, so that the electron scattering data can be interpreted with a single scattering theory. Theoretically, the specimen thickness should not exceed about 700 Å if the targeted resolution is 3.5 Å with a 400 kV microscope. Beyond this specimen thickness, the phase error of the structure factors might approach 90° at that resolution. An added advantage of using higher electron energy is to reduce the chromatic aberration effect, resulting in a better-resolved image (Brink & Chiu, 1991).

There are different types of electron emitters, including tungsten filaments, LaB_6 crystals and field emission guns, all of which use different mechanisms to generate the electrons. The field emission gun produces the brightest, most monochromatic beam. The high brightness can allow the electrons to be emitted as from a point source to irradiate the specimen with a highly parallel (*i.e.* a highly spatially coherent) illumination. The benefit of high spatial coherence is the preservation of high-resolution details in the image, even though the defocus of the objective lens is set very high in order to have low-resolution feature contrast (Zhou & Chiu, 1993). Thus, a field emission source is the best choice for high-resolution data collection.

The recording medium of an electron microscope can be an image plate, a slow-scan charge-coupled-device (CCD) camera, or photographic film. Because of their broad dynamic range and high sensitivity, both the CCD camera and the image plate are best suited for recording diffraction patterns (Brink & Chiu, 1994). However, for high-resolution image recording – when the recorded area, pixel resolution, signal-to-noise ratio and the modulation transfer function characteristics must be considered – photographic film is the optimal choice (Sherman *et al.*, 1996).

19.2.3. Data collection

19.2.3.1. *Specimen preparation*

An electron microscope column is kept at a pressure of $< 10^{-6}$ Torr (1 Torr = 133.322 Pa). Because a thin protein crystal loses its crystallinity if dried in a vacuum, its hydration can be maintained by embedding it in a thin layer of vitreous ice, glucose, or other small sugar derivatives (Unwin & Henderson, 1975; Dubochet *et al.*, 1988). The effectiveness of these preservation methods is evidenced by the high-resolution diffraction orders (out to at least 3 Å) from properly embedded protein crystals (Fig. 19.2.3.1). Since the high-resolution reflections come mostly from the protein, their diffraction intensities are largely independent of the embedding medium. However, the low-resolution diffraction intensities can be affected by the embedding medium because different media have different scattering densities relative to the protein. For any new crystal, any of the embedding media mentioned above can be used for high-resolution structural studies.

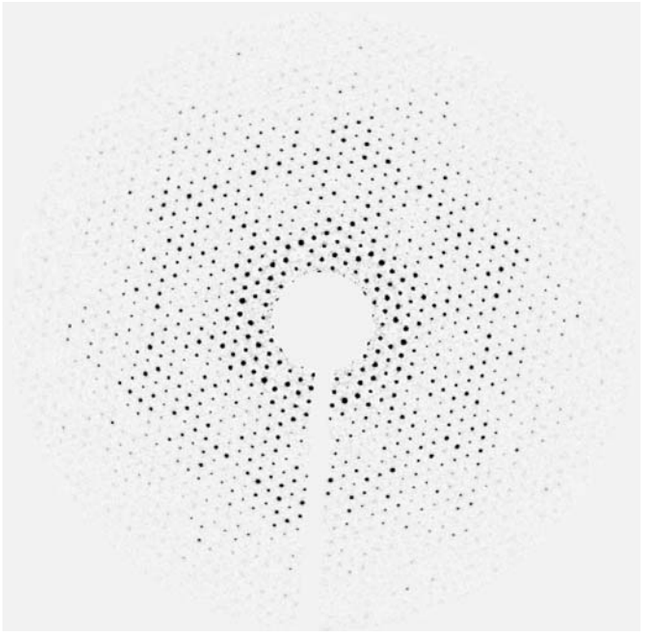

Fig. 19.2.3.1. Electron diffraction pattern of trehalose-embedded bacteriorhodopsin, with Bragg reflections extending to 2.5 Å. The unit-cell parameter of this 45 Å-thick membrane protein crystal is 62.5 × 62.5 Å arranged in a *p*3 two-dimensional space group. The raw diffraction pattern was recorded on a Gatan 2k × 2k CCD camera with 300 kV electrons in a JEOL 3000 electron cryomicroscope equipped with a field emission gun and a liquid-helium (4 K) cryoholder. The pattern displayed has been contrast-enhanced using radial background subtraction. A central beam stop was used to prevent saturation of the detector but has blocked off some reflections. R_{sym} for the Friedel-symmetry-related reflections (about 290 pairs) was computed to be about 5%. (Courtesy of Drs Yifan Cheng and Yoshinori Fujiyoshi at Kyoto University.)

19.2.3.2. *Radiation damage*

All protein crystals are prone to radiation damage caused by inelastically scattered electrons (Glaeser, 1971). This physical process is easily seen in the fading of electron diffraction intensities of a protein crystal as the accumulated doses increase. The consequence of damage is a preferential loss of the high-resolution information. Radiation damage is a dose-dependent process and cannot be reduced by adjusting the dose rate (flux) of the irradiating electrons. The strategy used to minimize the damage is to record the diffraction or image data from a specimen area that has not been previously exposed to electrons for purposes of focusing or other adjustments (Unwin & Henderson, 1975). This is called a minimal or low-dose procedure. In addition, keeping a specimen at low temperature (<113 K) allows it to tolerate a higher radiation dose (by a factor of about 4 to 6) before reaching the same extent of damage as at room temperature (Hayward & Glaeser, 1979). It has been shown that damage reduction is minimal below liquid-nitrogen temperature (Chiu *et al.*, 1981). However, there have been some impressive results using the electron cryomicroscope to study membrane protein crystals kept at liquid-helium temperature (4 K) (Kühlbrandt *et al.*, 1994; Kimura *et al.*, 1997; Miyazawa *et al.*, 1999).

19.2.3.3. *Other technical factors*

In order to record a three-dimensional data set, the crystals have to be tilted to different angles with respect to the direction of the electron beam. In a typical electron microscope, the highest angle to which the specimen stage can be tilted is about ±60°. Consequently, there is a missing set of data beyond the highest tilt angle, which corresponds to no more than 15% of the entire three-dimensional volume. Because of the radiation damage, a single diffraction pattern or a single image per crystal is usually recorded (Henderson & Unwin, 1975). The quality of a crystal is easily judged by its electron diffraction pattern as captured from a CCD camera during data collection. Evaluating the ultimate quality of images, however, takes more time and requires extensive computational analysis.

There are two major technical problems that often limit the data quality, even though a crystal is highly ordered (Henderson & Glaeser, 1985). One is the flatness of the crystal, and the other is the beam-induced movement or charging of the crystal. The effects of both problems become more prominent when the crystals are tilted to high angles. These effects tend to blur the diffraction spots, resulting in loss of high-resolution data (Brink, Sherman *et al.*, 1998). There are many ways to overcome these technical handicaps. For instance, the type of microscope grid chosen or the method of making the carbon support film is critical for reducing the wrinkling of the crystals (Butt *et al.*, 1991; Glaeser, 1992; Booy & Pawley, 1993). The use of a carbon film, which is a good conducting material, to support the protein crystal appears to reduce specimen charging (Brink, Gross *et al.*, 1998). It has been suggested that using a gold-plated objective aperture is effective in reducing specimen charging by generating a stream of secondary electrons to neutralize the positive charges that have built up on the specimen, which thus acts like an aberration-inducing electrostatic lens. Empirically, irradiating the microscope grid before depositing the specimen also reduces the charging (Miyazawa *et al.*, 1999). All these technical problems that can hamper progress in the completion of the structure determination have gradually been identified and resolved. However, more convenient and more robust experimental procedures for reducing these effects further are desirable in order to enhance the efficiency of data collection.

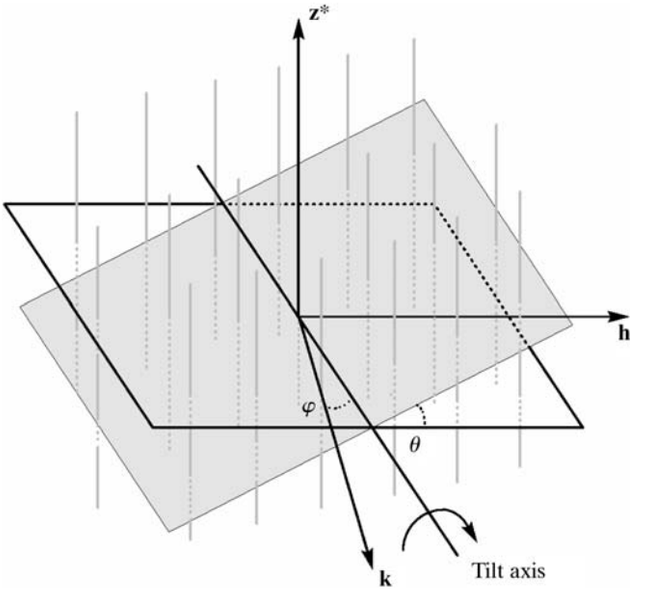

Fig. 19.2.4.1. Schematic diagram of data distribution in Fourier space for a two-dimensional crystal. Both the amplitudes and phases of the structure factors are distributed along the lattice lines passing through the (*h*, *k*) projection plane. The sectional plane shown denotes data from a crystal tilted at a certain angle (φ), and its tilt axis has an inclined angle (θ) with respect to one of its crystallographic axes. (Courtesy of Dr Jaap Brink at Baylor College of Medicine.)

references