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HIGH RESOLUTION ELECTRON CRYSTALLOGRAPHY OF PROTEIN MOLECULES

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ABSTRACT

Electron diffraction data and high resolution images can now be used to obtain accurate, three-dimensional density maps of biological macromolecules. These density maps can be interpreted by building an atomic-resolution model of the structure into the experimental density. The Cowley-Moodie formalism of dynamical diffraction theory has been used to validate the use of kinematic diffraction theory (strictly, the weak phase object approximation) in producing such 3-D density maps. Further improvements in the preparation of very flat (planar) specimens and in the retention of diffraction to a resolution of 0.2 nm or better could result in electron crystallography becoming as important a technique as x-ray crystallography currently is for the field of structural molecular biology.

Running title: Protein Crystallography

1. Introduction

It has always been a goal of biological users of the electron microscope to take advantage of the high resolution provided by this instrument to obtain images of biological macromolecules in atomic detail. A major step towards this goal occurred about 25 years ago, when the conceptual and mathematical approach of crystallographic structure analysis, already a mainstay of electron microscopy in the materials sciences [1], was introduced to biological electron microscopy [2, 3, 4]. The practical realization of this goal, i.e. high resolution structure determination of proteins and other biological macromolecules, faced a number of potential difficulties, however, that were either obvious from the start, or soon became self-evident, such as the need to work with hydrated specimens in the vacuum of the electron microscope, the severe limitation in electron exposure that could be tolerated due to radiation damage, and the worry that multiple scattering would render the convenient Fourier transform relationships of kinematic scattering theory invalid for electron crystallographic structure analysis. These problems, and many others that emerged as the research went forward, have now been largely resolved, and the experimental option of electron crystallographic structure analysis is finally beginning to emerge as a practical method for solving the structure of biological macromolecules at atomic resolution.

Bacteriorhodopsin, a bacterial cell-membrane protein [5], is the first biological structure for which a high resolution molecular model has been built, based upon electron crystallographic data [6]. While the resolution achieved in the 3-D density map was anisotropic, due to the difficulty in obtaining data at tilt angles higher than 45 degrees, the resolution in the best directions, parallel to the membrane plane, was good enough (.28 nm) that the known amino acid sequence of this protein could be fitted quite unambiguously into the density map. Good crystals of two other membrane proteins have also led to high resolution pictures of the organization of their secondary structure [7, 8], and additional work, not yet published, indicates that even higher quality chain-trace interpretations will be obtained

for these structures than was true for the first structure, bacteriorhodopsin. Even more recently, good crystals of tubulin have resulted in a high resolution, 2-D projection of this structure [9], and this specimen, too, has promise to result in a 3-D structure at atomic resolution.

The technical capabilities of electron crystallography of biological macromolecules are now sufficiently well established, by the successes described above, that it is worthwhile to take a broader look at the role that electron crystallography can play in structural molecular biology. To set the context for this overview, we first review those physical principles of the method that distinguish it from x-ray crystallography. We next address some of the key physical concerns that are specific to the method, such as specimen flatness, dynamical diffraction effects, and the problem of anisotropic resolution in three dimensions. We then conclude with a summary of the kinds of problems in cell and molecular biology where it appears that electron crystallography now has the power to reveal molecular structure in atomic detail.

2. Role of electron crystallography in structural biology

The extraordinary strength of electron scattering, about 10^5 to 10^6 times that of x-rays, is the dominant physical characteristic that both enables and - ultimately - constrains the unique role of electrons for crystallographic structure analysis. Not to be forgotten, however, is the ability of electrons to be focused, and thus to give images of the scattering object at resolutions that can now be well below 0.2 nm, when limited only by instrumental performance. The high scattering power of electrons makes it possible to get more than adequate signal from single-crystal specimens of biological macromolecules that are only one unit cell thick, such as the crystals that are produced by membrane proteins within their natural, lipid bilayer environment. Single-crystal specimens that are used for x-ray crystallography, on the other hand, are typically much more than 10^4 unit cells in thickness. The difference in scattering power therefore opens up a whole realm of structural questions, involving extremely thin specimen materials, that are completely inaccessible to other

methods. The availability of real-space images is an additional, powerful feature of electron crystallography, providing direct access to the crystallographic phase information. Electron crystallography therefore has no requirement to use isomorphous heavy atom derivatives in order to solve the phase problem, a step that can still be a major limitation in x-ray crystallography. On the other hand, the requirement to work with exceedingly thin specimens carries with it the danger that they are easily buckled or wrinkled to such an extent that it becomes impossible to collect data at high tilt angles, unless a way around the wrinkling is found, as will be explained further in the next section. Thus, for each new specimen, electron crystallography will generally have to solve the "specimen flatness" problem, appropriate to that specimen, while x-ray crystallography must in each case solve its "heavy atom derivatives" problem.

Structure determination by electron crystallography is now able to produce 3-D density maps at a high enough resolution that they can be interpreted in terms of the atomic structure of the protein, just as is the case for density maps obtained in x-ray crystallography. The initial fitting of the polypeptide chain to the 3-D density map is only the first step in a high resolution structure analysis, however. The next step in x-ray crystallography is to refine the structure by minimizing the difference between the observed diffraction intensities and the intensities calculated for the atomic resolution model of the structure. The refinement step is needed in x-ray crystallography because it normally is difficult to get phase information from isomorphous derivatives to a resolution much higher than 0.35 nm, and the procedure works well because the number of higher resolution, unphased reflections can easily be equal to, or exceed the number of reflections that went into the original 3-D density map. The situation in electron crystallography is inferior to x-ray crystallography at present, in that specimen preservation is still relatively poor, and crystals rarely diffract to much better than 0.35 nm. The outlook in the long term is promising, however, in that accurate phase information, as well as diffraction intensities, should be obtained from images at 0.2 nm resolution as easily as at 0.35. With improvements in specimen preparation, then, the quality of structure determinations

that would be possible with such high resolution image data would actually exceed the already high standard that has become common for *refined* x-ray structures.

The direct access to phase information that is available in electron crystallography also holds out the possibility to solve much larger structures than is possible by x-ray crystallography. X-ray structure determination of high molecular weight structures is limited by the proportionately smaller effect that a few heavy atoms can have on modifying the native diffraction intensities. Adding more heavy atoms would, of course, increase the phasing power of the derivative, but that approach becomes impractical when the number of heavy atoms becomes too numerous, because the initial difference Patterson map becomes impossible to solve. The ability to retrieve high resolution phases from high resolution images is the same for any structure, however, independent of size, as long as the number of unit cells in the image is the same. There is also the tantalizing possibility, to be mentioned further in the last section of this paper, that it may be at least as easy to obtain well-ordered crystals of very large structures by monolayer techniques as it is to obtain 3-D crystals of the same structures.

3. Specimen flatness

In order for the Fourier spectrum of a monolayer crystal to remain sharp and discrete at high tilt angles, the specimen must remain flat (planar) to a tolerance of better than one degree. Imperfection in the specimen flatness causes broadening of the diffraction spots that lie in the direction perpendicular to the tilt axis, ultimately causing them to blur together. Bending or wrinkling also leads to the superimposition of nonequivalent information along the continuous reciprocal lattice lines [10]. The influence of crystal bending upon diffraction intensities, compared to those of an unbent crystal, and the effect of bending on coherent diffraction from different successive layers within the crystal, have also been treated by Cowley from a real-space (Patterson function) perspective [11]. Imperfect specimen flatness can therefore be a significant factor in limiting the highest tilt angle at which data can be obtained, as we have already pointed out above.

The experimental procedures that will ensure adequate specimen flatness are still not fully understood. Monolayer crystals of biological macromolecules are all likely to be too flexible to remain unbent, on their own [10]. A number of different experimental schemes, or physical situations have been proposed under which adequate flatness might, in principle, be achieved [10], but most such strategies have not yet been investigated in a systematic fashion.

The best characterized method of specimen mounting remains the technique of embedding the crystalline specimen in glucose (or another, equivalent, hydrophillic embedment), on a thin carbon film. Even this technique is only very poorly understood, however. It seems that the specimen flatness, and how well-ordered the specimen remains after embedment, depends very much on the surface properties of the carbon film. Unfortunately, these surface properties can vary greatly from one day's preparation of carbon film to another, from one laboratory to another, and over prolonged aging of the same preparation. Even so, extremely flat, well-preserved specimens can be obtained after some trial and error, not unlike that which is involved in the search for heavy atom derivatives in x-ray crystallography.

In our work with bacteriorhodopsin we have encountered the further complication that specimens which initially are extremely flat, at room temperature, almost always become badly wrinkled when cooled to -100 C or lower. Fortunately, however, the problem of cooling-induced wrinkling can be overcome by using molybdenum grids rather than copper grids [12].

Encouraged by the improvement in quality of electron diffraction patterns at high tilt angles, we have now confirmed that the use of molybdenum grids also results in a very big improvement in our ability to obtain high resolution images at tilt angles up to 60 degrees. Provided that the electron diffraction pattern of the crystal is itself very sharp perpendicular to the tilt axis, we find that the Fourier transform of the image can have a signal-to-noise ratio that is nearly as good in the direction perpendicular to the tilt axis as it has parallel to the tilt axis. Figure 1 shows a representation of one such Fourier transform, in which the signal-to-noise ratio at each reciprocal lattice point is represented by a line segment whose

length increases in proportion to a quantitative measure of the quality of the data, as defined by Henderson et al. [13]. The number of strong electron diffraction spots is always much smaller perpendicular to the tilt axis for bacteriorhodopsin, and therefore it is expected that the number of good reflections in the computed Fourier transform will also be smaller. But, as can be seen in Figure 1, the highest values of the signal-to-noise ratio do not drop appreciably in the direction perpendicular to the tilt axis. The use of molybdenum rather than copper grids therefore appears to be a small but technically important detail for work at low specimen temperatures, at least when specimens are supported on thin carbon films.

4. Dynamical diffraction effects

The extremely strong scattering of electrons has quite properly been a matter of concern in relation to the use of the weak phase object approximation as the theoretical basis for structure analysis in electron crystallography. Because there is no known, generally applicable mathematical inverse within dynamical diffraction theory, and because the structure of biological macromolecules is too complex to use image-matching calculations for *de novo* structure determination, the future of the whole field of high resolution electron microscopy of biological macromolecules was potentially at risk. The Cowley-Moodie formalism of dynamical diffraction theory [15] has made it possible, however, to make quantitative comparisons between kinematic and dynamical theory, and these comparisons have fortunately provided a sound justification for the use of the weak phase approximation. Although the kinematic approximation and the weak phase approximation are not identical, of course, we will refer to them interchangeably for the purpose of discussion in this paper. All actual comparisons reported here involve, in practice, only the weak phase object approximation.

Starting first with organic crystals of relatively simple structure [16], and progressing subsequently to the case of a known protein-crystal structure [17], dynamical theory calculations were used to map out the domain of specimen thickness, resolution, and accelerating voltage within which the usual kinematic theory remained valid to a specified

degree of accuracy. The conclusion drawn from these numerical experiments is that the kinematic theory can be used for unstained biological macromolecules up to a specimen thickness of at least 10 or 20 nm, for voltages of 100 kV or more. A similar conclusion has been reported more recently, in an independent simulation [18].

Experimental support for the negligible influence of dynamical effects has also been found in the measurement of violations of Friedel symmetry in electron diffraction patterns recorded from glucose-embedded crystals of bacteriorhodopsin [19]. Systematic Friedel differences as large as 40 per cent can be seen in diffraction patterns recorded at 20 kV, but at 120kV all except a few of the Friedel differences are less than 10 per cent, which is almost too small to be reliably measured.

In new work reported below, we have further investigated the magnitude of dynamical diffraction effects, including the violation of Friedel symmetry, in electron diffraction from bacteriorhodopsin (bR). In order to carry out these calculations we have used the atomic model of bR [6] as input for dynamical diffraction calculations according to the Cowley-Moodie multislice formalism. The computer package NCEMSS [20] was used, in which the unit cell structure of bacteriorhodopsin (bR) was represented as a layered structure, each successive layer being composed of the atoms of the bR model structure that actually do lie within successive, 0.5 nm thick slices. In order to explore a greater range of specimen thicknesses than the ~4 nm thickness of the bacteriorhodopsin molecule, the calculations were carried out for samples that were up to five unit cells in thickness. The resolution limit in the calculation was set at 0.16 nm, which means that diffraction orders to twice that resolution were actually used for the calculations. However, experimentally determined temperature factors, which are included as part of the structural information in the Brookhaven Protein Data Bank, were retained in the calculation of the scattering potential; the effect of doing this will be to suppress the influence of reflections at excessively high resolution, and which would not be present in diffraction from the real bacteriorhodopsin crystals. The calculations reported here were all carried out for 100kV electrons, and the results are analyzed within the resolution

zone from 2 to 0.35 nm, typical of the range of electron diffraction data that are available in current work with biological macromolecules. The results obtained are summarized in Table 1.

The existence of noticeable dynamical effects is seen even for a thickness of 4 nm (one unit cell), if one compares the diffraction intensities that are calculated for Friedel pairs. The R-factor between Friedel mates, for 100 kV electrons, is already 11% at 4 nm thickness, and grows to over 40% at 20 nm thickness. At the smallest thickness (4 nm), however, the impact of these dynamical effects will be extremely small, because the R-factor between the average value of the Friedel mates on the one hand, and the corresponding value calculated from the weak phase approximation is only 1%. The intensity-weighted root mean square (rms) phase difference between the dynamical phases and those obtained from the weak phase approximation, even without averaging Friedel mates, is small, only about 5 degrees, and less than 0.1% of the power in the diffraction pattern is associated with reflections for which the dynamical phases differ from those derived from the weak phase approximation by more than 30 degrees. Each of these indicators of dynamical "distortions" in the data becomes progressively worse, of course, as the specimen thickness increases, as can be seen in Table 1. Setting the Friedel R-factor aside for the moment, none of the indicators of dynamical effects become large enough to be considered a significant effect until the specimen thickness is at least 12 nm. These calculations support earlier conclusions, cited above, that dynamical effects can be ignored, and kinematic diffraction theory can be used to solve crystal structures of biological macromolecules up to a specimen thickness of 10 to 20 nm, using data obtained with 100 kV electrons.

Although Friedel differences in amplitude appear to be a surprisingly sensitive indicator of dynamical diffraction, these differences turn out to cancel one another to first order, when Friedel mates are averaged, as is done with real data. The simulations reported in Table 1 therefore show that the weak phase object approximation can be used with little error to interpret diffraction intensities which are the average of Friedel mates, for suitably thin specimens.

5. The cone of missing data

For simple geometric reasons it is impractical to collect data from thin, sheet-like specimens that are tilted to angles much more than 60 degrees, and, as discussed above, imperfect specimen flatness also places a definite limitation on the maximum tilt angle for which one can collect high resolution data. These limitations on tilt angle in turn mean that there is a conical volume of reciprocal space within which it is not possible to measure the experimental structure factors. Three-dimensional reconstructions in electron crystallography therefore must, in general, be carried out with incomplete data, although there are special cases (for example, helices) in which the point group symmetry of the unit cell makes it possible to collect the full set of unique data within a relatively small range of tilt angles.

Even in the "worst case", however, corresponding to a missing cone of data as large as 30 degrees (i.e. specimen tilting up to 60 degrees), less than 14 per cent of the volume of reciprocal space is excluded from measurement. More importantly, however, calculation of the 3-D point-spread function shows that the resolution in the direction perpendicular to the plane of a 2-D crystal will be worse by only a factor of ~1.3 relative to the resolution in the direction parallel to the plane of the crystal [21, 22].

If the resolution of the 3-D reconstruction is high enough, the effect of this anisotropic point spread function should have little effect on the ability that one will have to correctly interpret the 3-D density map. Formally, one could argue that the 30 per cent reduction in resolution (in the direction of the missing cone of data) should be compensated by collecting data to 30 percent higher resolution than would be needed to obtain an interpretable map at isotropic resolution, and in some instances it may be possible to do this. In practice it appears that collecting higher resolution data will not even be necessary, judging from a simulation that has been done with a protein of known structure [22]. In this simulation, a missing cone was applied to the 3-D Fourier transform of an atomic model of the protein monellin. The maximum resolution was then limited to 0.36 nm, a value commonly found to be sufficient to permit interpretation in terms of an atomic resolution model, and contour maps at different

levels of the structure were compared before and after applying the missing cone. The difference in the density maps was found to be much too small to alter the interpretation, regardless of the direction of the axis of the missing cone with respect to the molecular coordinate system, and without regard to the inclusion of additive noise.

6. Future opportunities

Having reviewed the fact that electron crystallography is now a practical method for structure analysis of biological macromolecules, as judged from the point of view of relevant theoretical considerations as well as present-day experimental results, it is worthwhile to conclude by looking a bit into the future, speculating on the opportunities that exist for preparing thin crystals that are suitable for this method of structure analysis.

Integral membrane proteins, which exist in nature in a form that is inserted into and spans across a lipid bilayer, represent one major class of structures that seems to be especially well suited for 2-D crystallization. Bacteriorhodopsin is an example in which the protein even crystallizes spontaneously within the plane of the native cell membrane, and a few other examples of such behavior are known. In most cases, however, it will be necessary to first purify the desired protein in a detergent-solubilized state, and then attempt to get 2-D crystals by reconstituting the protein with a limiting amount of lipid [23, 24, 25]. Although some detergent-solubilized integral membrane proteins can also be crystallized in three dimensions, in a form suitable for x-ray diffraction [26, 27, 28], there are many other cases where that approach has not yet been successful. Electron crystallography now offers a second, independent opportunity to achieve a high resolution structure analysis of this important class of proteins.

In some cases, even soluble proteins may resist the effort to find conditions that give well ordered, three-dimensional crystals. Some proteins of this type do make thin, well ordered crystals, however, and in those cases structural studies can now proceed by electron crystallography. High resolution diffraction and microscopy with thin plates of crotoxin [29]

and the recent work on 2-D crystals of tubulin [9] are good examples of what is possible in this direction.

Specific binding at an aqueous interface [30] and even nonspecific adsorption to an aqueous interface may both provide a more systematic approach by which one can attempt to obtain 2-D specimens that are suitable for electron crystallography [24]. The potential that this approach offers for high resolution structure analysis was demonstrated by Kubalek et al. [31], who crystallized streptavidin by allowing it to bind to a small ligand, biotin, which was attached covalently to lipid and spread at an air-water interface. After transfer to an electron microscope grid, followed by glucose embedding, these crystals were shown to diffract to 0.3 nm resolution. The work with streptavidin is just a prototype for a very large number of other cases where the natural ligand or substrate for any chosen protein can be covalently attached to the polar head-group of a lipid, and used as "bait" to bind the protein to the interface at a high enough concentration that 2-D crystals may begin to form. The potential effectiveness of interfacial crystallization methods has been further illustrated by the demonstration that some macromolecules will form 2-D crystals on the face of a positively charged lipid monolayer. First demonstrated for RNA polymerase [32], this simple technique has also yielded large, monolayer crystals of alpha-actinin [33].

Interfacial adsorption (binding) and crystallization represent an approach that could well become the single most productive technique for obtaining the thin crystals that are needed for structure analysis by electron crystallography. Even in cases where growing 3-D crystals could in principle be an option, interfacial crystallization may one day become the method of choice because of the simplicity of the method, its requirement for only very small amounts of protein, and the fact that there is no need to search for heavy atom derivatives in order to phase the diffraction intensities. The development of methods for mounting monolayer crystals with nearly perfect flatness, and the development of methods that preserve the crystalline order of the specimen to a resolution of 0.2 nm would ensure that the resulting structure determinations would match, or even exceed the standard that is found in present-day

applications of x-ray crystallography. Only modest improvement in specimen preparation techniques may therefore result in an explosive adoption of the method of electron crystallography in structural molecular biology.

7. Conclusions

The methods of high resolution electron diffraction and electron microscopy, here referred to as "electron crystallography," have now been developed in the context of biological macromolecules to the stage that three-dimensional density maps can be interpreted at the level of fitting a known amino acid sequence to the density. Imperfect specimen flatness can still be a limitation in such work. A real improvement in high tilt image data will be achieved, however, for low-temperature applications when care is taken to ensure that the thermal expansion coefficient of the support grid does not exceed that of the supporting carbon film. Theoretical concerns that were discussed in the stage before high resolution structures had been achieved experimentally included (1) limitations due to dynamical diffraction effects and (2) the limitations in three-dimensional information that are associated with finite limitations on the tilt angles over which data can be collected. Dynamical effects do not seem to significantly affect the data analysis for specimens that are less than 10 nm to 20 nm thick, and the cone of missing data does not limit the structural interpretation of the density map if the resolution of the map is high enough. All methodology is now in place to apply and further develop this new tool for molecular structure analysis in areas of structural biology where very thin, two-dimensional crystals can be obtained, thus providing access to many types of research problem that previously could not be addressed by other tools of molecular structure analysis.

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FIGURE LEGENDS

Figure 1. Graphical representation of the signal-to-noise ratio at the various reciprocal lattice points in the calculated Fourier transform of an image of a bacteriorhodopsin crystal that was recorded at a tilt-angle of 55 degrees. At points represented by dots, the signal is less than the average background, while line-segments of increasing length indicate increasingly greater signal-to-noise ratios, according to the scheme of Henderson et al. [13]. The reciprocal lattice in these crystals is always much more densely populated with strong reflections in the direction parallel to the tilt axis than it is perpendicular to the tilt axis, as can be seen in electron diffraction patterns of highly tilted specimens. However, as this image shows, the use of improved imaging conditions, improved data analysis, and molybdenum support grids now makes it possible to obtain equally good values of the signal-to-noise ratio at high resolution, in all directions. This image was recorded with spot-scan illumination and dynamic focus [14], and appropriate modifications were made in the processing of such images to remove a residual defocus ramp within each illuminated spot as well as to unbend distortions across the entire image, as described by Henderson et al. [13].

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