117·6 (4), (4) 117·4 (2), (5) 117·4 (1)°. The P—O bond lengths increase from (2) 1·551 (3) Å to (5) 1·586 (2) Å (Table 6) in line with increasing electron supply. This is reflected in a decrease in the three-bond spin–spin coupling constant, $^3J(POCC)$, which varies from (2) 7·35 Hz to (4) 6·45 Hz and to (5) 5·9 Hz. The average POCC dihedral angles change from (2) 54·4° to (4) 56·3° and (5) 57·2°. If no factors other than dihedral angles affected the vicinal coupling constants, a marginal overall decrease of 0·3–0·4 Hz would have been expected. The considerably larger changes observed must be attributed to the P—O bond lengthening with increasing electron supply to the P atom.

The decrease in $^3J(POCC)$ and $^3J(PNCC)$ spin–spin coupling constants with increased P—O and P—N bond lengths, respectively, indicates the importance of Fermi contact. The large Fermi contact contributions to coupling constants in cyclophosphazenes have been established experimentally and theoretically by Thomas, Grossmann & Meyer (1981).

$^3J(POCC)$ spin–spin coupling constants are widely used to obtain information about conformation; this includes molecules of biological importance. Our results show that factors other than dihedral angles affect vicinal coupling constants, and these must be taken into consideration. However, in most other phosphorus systems, changes in electron supply towards the P—O bond will be much less than in phosphazene derivatives, hence changes in P—O bond lengths would be smaller and therefore more difficult to observe.

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Cryocrystallography of Biological Macromolecules: a Generally Applicable Method

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Abstract

Methods have been developed that allow facile X-ray data collection for biological macromolecules at cryogenic (near liquid N_2) temperatures. The crystals are first transferred from their mother liquor to a hydrocarbon environment, then mounted with a standard glass fiber (no capillary), and flash cooled *in situ*

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with a cold nitrogen stream on the diffraction apparatus. This approach prevents freezing of the solvent in the crystals, so that they maintain their crystallographic integrity. Significant improvement of resolution can result from the cryogenic data collection, and radiation damage in the cooled crystals is greatly reduced, or eliminated, for the duration of data collection.

Introduction

Crystals of biological macromolecules are notorious for their sensitivity to X-ray irradiation, and for the rapid decrease in diffracted intensity with increasing $(\sin\theta)/\lambda$. Radiation damage can lead to serious experimental problems. It is often necessary to use a number of crystals in order to record a full data set. Cases where sample decay prevents successful data collection are not rare. Data from radiation-damaged crystals in general will not be as reliable as data from stable ones. The preparation and mounting of numerous samples can seriously tax the resources of a research group.

The decrease in intensity with increasing diffraction angle is mainly connected with high B values, typically 10–20 $Å^2$ or higher, and with the fact that radiation damage first tends to affect the higher-resolution data. Experience from a large number of small-molecule studies in this laboratory shows a dramatic reduction in radiation damage and other decay during data collection, as a result of keeping the crystals near liquid-N_2 temperature. A substantial increase in intensity is also seen at higher diffraction angles. Biological macromolecule crystals can reasonably be expected to show similar behavior.

It has been widely assumed that most biocrystals with high water content will be destroyed by internal ice formation on cooling below the freezing point. However, a number of reports have indicated that by selecting appropriate conditions, it is possible to cool biocrystals to cryogenic temperatures without damage.

Cooling experiments on insulin crystals for the purpose of reducing radiation damage have been described by Low, Chen, Berger, Singman & Pletcher (1966). Although in some respects the experiments were successful, the authors were dissatisfied with the resulting mosaic spread, and inexplicably abandoned further cryogenic work. In retrospect it appears that this was a most unfortunate decision.

In order to avoid the problem of internal ice formation, Petsko (1975) has replaced the normal mother liquor with a salt-free aqueous-organic mixture of low freezing point. Crystals treated in this manner have withstood several cooling–warming cycles between room temperature and 153 K. The method has not found wide use. In addition to being difficult to perform, the technique also leads to the destruction of the natural solvent structure, with loss of potentially valuable information, except for cases where the solvent exchange is only a surface phenomenon.

Prevention of phase separation by immersion in liquid propane was reported by Parak, Mössbauer, Hoppe, Thomanek & Bade (1976). This method was used in a study of myoglobin at 80 K (Hartmann, Parak, Steigemann, Petsko, Ringe, Ponzi & Frauenfelder, 1982). Although successful, the method has not been used much, presumably because of its technical complexity. However, in view of the possibility of very rapid cooling, further work on this method seems desirable.

In a study of a B-DNA dodecamer, Drew, Samson & Dickerson (1982) used an extremely slow cooling process to cool a crystal to 16 K. Although no phase separation was observed, and the suggestion was made by the authors that protein crystals may be successfully cooled under similar conditions, no follow-up has been reported.

Clearly, there has been a need for improvement in the methods of data collection for biological macromolecular crystals. The ability to collect data at cryogenic temperatures can be expected to result in important improvements in quality of data, speed of data collection and amount of information obtained from a structure determination. Development of the necessary methods was therefore initiated in this laboratory. The resulting simple, generally applicable procedures are described in this article.

Crystal handling

Biological macromolecule crystals have traditionally been mounted in capillaries, together with some mother liquor, in order to avoid sample damage from loss of solvent. However, capillary mounting is often cumbersome, and in a cooling process the capillary can act as an insulator. For these reasons a technique for crystal mounting without the use of capillaries was developed. The method involves the use of an inert oil that serves both as a protective shield for the crystal during handling, and as an adhesive upon cooling. Cooling is effected with a cold-gas-stream apparatus. For best results it appears that the cooling process should be as quick as possible. A brief description of the method as applied to small-molecule crystals has been given earlier (Hope, 1985). The oil used in these experiments is Paratone-N (Exxon), mixed with mineral oil to attain suitable viscosity (typically 25 to 50% mineral oil). Presumably other products with similar characteristics would be equally useful.

The procedures involved in transferring a crystal from its mother liquor to the cold stream on a diffractometer can be divided into three steps: (*a*) transfer of the crystal from its mother liquor to a hydrocarbon environment; (*b*) transfer to a crystal mounting pin; (*c*) cooling to cryogenic temperature.

Transfer from mother liquor to oil

Bio-crystals are grown by a variety of techniques: *e.g.* from bulk solution, in a hanging drop, or in a sitting drop. The transfer technique depends in part on the method of crystallization employed.

Drop-grown crystals result in the simplest technique. The coverslip holding the drop is placed with the drop side facing up. The drop containing the crystal is then completely covered with a large (0·5 ml or so) drop of the inert oil. With a needle or a razor blade the crystal, together with a small amount of mother liquor, is moved into the oil. The mother liquor adhering to the crystal is then removed. This can be done with the tip of a strip of dense filter paper cut to form a point, or by just moving the crystal around in the oil. Since the mother liquor most likely will freeze on cooling, it is important to remove as much as possible of it from the surface of the crystal, in order to avoid mechanical damage, or interference from the ice powder pattern. Because the oil is viscous and hydrophobic the loss of water from the crystal into the oil is slow, and most crystals will not suffer any rapid damage from this treatment. It is still prudent to work without unnecessary delay at any stage.

Crystals grown in bulk solution can conveniently be transferred to a drop of oil with a pipette. The crystal, with a small amount of mother liquor, is sucked into the pipette tip in the usual way, and then injected into the oildrop. Following this the procedure is the same as for drop-grown crystals.

Transfer to the mounting fiber

As soon as the crystal is sufficiently free from mother liquor, it should be transferred to the cold stream. A standard small-molecule-type glass fiber of appropriate length and thickness, attached to a metal mounting pin, can be used to lift the crystal from the oil. Typically the length of the fiber will be slightly less than the diameter of the cold stream, and the thickness about one-quarter the diameter of the crystal. Sometimes it can be difficult to move the crystal across the oil–air interface. Holding the fiber directly below the center of gravity of the crystal, and moving steadily, but quite slowly may help. One should attempt to make the adhering oil drop as small as possible by letting it drain off as it is pulled out of the oil.

Some crystals are so easily deformed that they will be damaged by this technique. A micro-spatula fashioned by gluing a small piece of flat, extremely thin glass to a mounting fiber can be used in such cases to hold the crystal. The thin glass can be obtained by blowing a glass bubble from a piece of glass tubing.

Transfer to the cold stream

The transfer into the cold stream should be carried out as quickly as possible. The best procedure, both in terms of rapid cooling and ice prevention, is first to temporarily deflect the cold stream while the crystal is being brought near its final position on the diffraction apparatus, and then with the crystal in place suddenly restore the flow of the cooling gas. The crystal is then ready for use.

The cold stream can be controlled quite simply by holding a piece of stiff paper, or a microscope slide, across the nozzle so that the cooling gas is deflected. Quick removal of the obstruction will result in sudden lowering of the temperature again. It is not advisable to shut off the stream at its source, since that will give rise to less-abrupt cooling.

Test cases

Crambin

The first cooling experiments by the techniques described above were performed on crambin, a relatively dry small protein (Hendrickson & Teeter, 1981). A number of crystals were cooled to 130 K, and evaluated by diffractometer methods. The equipment used was a Nicolet $P2_1$ diffractometer with a graphite monochromator and LT-1 cooling attachment, locally modified. Details of the modifications will be reported elsewhere.

Crystal dimensions varied between 0·3 and 1·2 mm. All specimens retained their crystallographic integrity. Typical full ω-scan widths were no wider than 1·2° base to base, similar to room-temperature scans. The extent of mosaic spread appears to be associated with properties of individual crystals, rather than with the cooling process. Many reflections with intensity well above background were seen with Cu $K\alpha$ at $2\theta = 135°$. An ω-scan data set (Cu $K\alpha$) to $2\theta_{max} = 138°$ (resolution $\lambda/2\sin\theta_{max} = 0{\cdot}83$ Å) was collected with a scan speed of 58·6° min^{-1} (Hope & Nichols, 1981), and backgrounds estimated from a sampling of about 200 points distributed over reciprocal space. The data collection time was 60 h. Of 32 000 measured reflections, about 80% had $I_{net} > 2\sigma(I_{net})$. Intensity stability was checked with two monitor reflections repeated every 400 reflections, and by remeasuring the first 2000 reflections at the end of data collection. There was no detectable change in intensity, indicating complete absence of radiation damage for the data-collection period.

The cell dimensions [130 (2) K] for the crystal used in the data collection are: $a = 40{\cdot}763$ (5), $b = 18{\cdot}492$ (3), $c = 22{\cdot}333$ (3) Å, $\beta = 90{\cdot}61$ (1)°, based on measurements with 2θ in the range 44–62°. Other crystals showed deviations up to ±0·06 Å from these values. The diffractometer and crystals had been carefully aligned for these experiments. It seems likely that the deviations reflect real differences between specimens grown under slightly different conditions. The cell dimensions at room temperature (~294 K) are

$a = 41{\cdot}08$ (2), $b = 18{\cdot}76$ (1), $c = 22{\cdot}72$ (1) Å, $\beta = 90{\cdot}52$ (4)° (Hendrickson & Teeter, 1981). The decrease in volume of about 3·5% as a result of the cooling is normal for molecular crystals.

The data set has been used in refinements of crambin. Perhaps the most intriguing result has been the precise determination of most solvent molecules, and the establishment of an extended hydrogen-bond network (Teeter & Hope, 1986). Apparently all solvent water in the crystal can be described as structural.

BPTI (bovine pancreatic trypsin inhibitor), form II

In terms of solvent content crambin is at the low end of the scale, and is therefore not representative of most protein crystals. A more typical example is BPTI, with about 40% solvent content.

Three different samples were cooled, all without apparent damage. The dimensions of these crystals were about 0·25 × 0·4 × 1·0 mm. Full ω scans were contained within 1°. Below $2\theta = 60°$ most intensities were observable, but at 105° only a few intensities could be measured above background. An ω-scan data set (Cu $K\alpha$) was measured with a scan speed of 58·6° min^{-1} for $0 < 2\theta \leq 60°$, 10° min^{-1} for $60 < 2\theta \leq 90°$, and 6° min^{-1} $2\theta > 90°$. Monitor reflections as well as remeasurement of the first 7000 reflections showed that there had been no observable radiation damage. The data set has not yet been used in refinements.

The cell dimensions [125 (2) K] are $a = 75{\cdot}39$ (3), $b = 22{\cdot}581$ (7), $c = 28{\cdot}606$ (9) Å, and the cell reported by Wlodawer, Walter, Huber & Sjölin (1984) is $a = 74{\cdot}1$, $b = 23{\cdot}4$, $c = 28{\cdot}9$ Å.

Expected effect of temperature on resolution

If B is an overall parameter, the intensity of a reflection is proportional to $\exp[-2B(\sin^2\theta)/\lambda^2]$. The dependence of resolution on temperature can be readily derived if the following assumptions hold: (*a*) the change in B is proportional to the Kelvin temperature, *i.e.* $B_T = B_0 + bT$, where B_T is the value of B at temperature T, B_0 is the 'zero-point' B, and b is a proportionality constant; (*b*) one can ignore changes in scattering factors and in the Lorentz and polarization factors with increasing diffraction angle; (*c*) at the limit of resolution the mean intensities are the same at the different temperatures. The relation between resolutions r_1 and r_2 ($r = \lambda/2\sin\theta$) at two temperatures T_1 and T_2 is then found from the equation

$$(B_0 + bT_1)/r_1^2 = (B_0 + bT_2)/r_2^2,$$

which merely equates two temperature-factor exponents. Expressing r_2, the resolution at temperature T_2, in terms of the other quantities one obtains

$$r_2 = r_1[(B_0 + bT_2)/(B_0 + bT_1)]^{1/2}.$$

If, for example, $T_1 = 300$, $T_2 = 100$ K, the highest resolution at 300 K is 3 Å, $B_0 = 5$ Å^2, and $b = 0{\cdot}05$ Å^2 K^{-1}, then the highest resolution at 100 K would be just over 2 Å. However, the assumptions made are likely to be somewhat crude; in addition, the effective resolution at the higher temperature may be reduced below that indicated by the B's of a fresh crystal, because of radiation damage. The resolution expression should therefore be taken only as a rough estimate of expected improvement. In any event, a substantial improvement in resolution can be anticipated for T_2 in the vicinity of 100 K.

Concluding remarks

The experiments described above indicate that cryocrystallographic measurements of biological macromolecules may be much more easily realized than has been previously assumed. Given a well functioning low-temperature apparatus, the procedures outlined here are operationally simple, and can be quickly learned. The main, and serious, obstacle in the way of general use is the installation and operation of an efficient frost-free cooling device. Commercially available equipment so far has suffered from deficiencies in design that demand a certain inventiveness and investment of time on the part of the user in order to make the equipment fully functional. The successful installation, and subsequent training, can therefore be costly in terms of laboratory resources. However, the potential for greatly increased data-collection efficiency, improved data, and savings in crystal-preparation effort should provide the necessary motivation to overcome initial difficulties.

The reduction in radiation damage alone can make the difference between a usable data set and one that does not yield interpretable results. Improved resolution and lower thermal parameters for crambin already have resulted in a much more detailed description of the solvent structure. It is likely that this result can be generalized, so that cryocrystallography carries the promise of better descriptions of protein–solvent interactions, with ensuing better understanding of the role of water in protein function.

Crystals mounted in capillaries, with or without their mother liquor, can give rise to strongly anisotropic absorption effects. Because of the difficulty in describing the geometry of the crystal environment it is virtually impossible to perform accurate analytical absorption corrections. Instead, one normally resorts to corrections based on φ scans of selected reflections. For a crystal encapsuled in a thin layer of hydrocarbon oil and mounted on a suitable glass fiber, overall absorption can be significantly reduced, and anisotropy will decrease. Because it will be possible to measure the dimensions of the crystal and the surrounding oil, more reliable absorption corrections become feasible.

Because the fluid holding the crystal becomes glass-hard at the low temperature, slippage of the crystal is completely eliminated. Redetermination of orientation matrices at the end of data collection revealed no observable change for the two test cases described above.

It is difficult to predict the effect of lower thermal motion on the process of structure solution. Normally a better data set leads to easier solution, but for some macromolecules it is conceivable that the greater visibility of the solvent in some way may interfere with the interpretation of electron density maps.

The observed variation in the cell dimensions of crambin point to a potential advantage in obtaining all data from one specimen. Crystals with slightly different cell dimensions will have slightly different structures, perhaps expressed as a difference in solvent arrangement. The averaging of data from different crystals can then result in smearing of electron densities in addition to that caused by thermal motion or disorder.

Dewan & Tilton (1987) have carried out low-temperature studies of ribonuclease, by use of a mounting technique first described by Hope & Power (1983). The crystals did not suffer phase separation, and virtual elimination of radiation damage was observed. Crystals of the same protein, mounted in coventional glass capillaries, were also successfully cooled; however, in this case the reduction in radiation damage was less pronounced. Although it would be of interest to determine the cause for this difference, it is not likely to be of immediate practical importance, since capillary mounting can be avoided. Available data do not appear to support the assumption of Dewan & Tilton that most protein crystals can be successfully cooled only after being transferred to a cryosolvent.

Further experiments in biological cryocrystallography are under way. Results will be described in forthcoming communications.

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Structure of Native Porcine Pancreatic Elastase at 1·65 Å Resolution*

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Abstract

The structure of native porcine pancreatic elastase in 70% methanol has been refined using film data to 1·65 Å resolution, $R = 0{\cdot}169$. A total of 134 molecules of water (but no methanol) has been refined. This structure, because of its native state and modestly high resolution, serves as the basis for comparison with other elastase structures complexed with natural or synthetic ligands. Internal structured water occupies distinct regions. Two regions (*IW*1 and *IW*7) suggest a mechanism for equalizing 'hydrostatic pressure' related

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