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Feasibility study for the measurement of a large set of triplet phases from a small protein

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The feasibility of measuring a set of triplet phases large enough to solve the structure of a small protein has been evaluated. A total of about 850 triplet phases have been measured from the tetragonal form of hen egg-white lysozyme. From these triplet phases, about 750 single phases can be derived. The experimental details of these measurements as well as the results, the values of the measured triplet phases, are reported. Additional experimental data from other small proteins are also presented.

1. Introduction

The first observations of three-beam interference profiles measured from a protein crystal were reported some time ago (Hümmer, Schwegle *et al.*, 1991; Chang *et al.*, 1991). Three-beam interference experiments with crystals of a number of other proteins followed (Weckert *et al.*, 1993). A recent comprehensive discussion of the theory of three-beam interference experiments and their application to various problems is given by Weckert & Hümmer (1997). The basics of three-beam interference experiments also are described in that paper and will not be repeated here. However, in order to facilitate the understanding of the following, some of these results will be summarized briefly.

It is well known that the phase of the structure factor of a reflection depends on the arbitrary choice of the origin of the unit cell. In an experiment, however, one can only determine quantities not depending on the choice of the origin: the structure invariants. This is the case in a three-beam interference experiment, in which one can determine directly an invariant triplet phase

$$\Phi_T = \varphi(\mathbf{g}) + \varphi(\mathbf{h} - \mathbf{g}) - \varphi(\mathbf{h}) \simeq \varphi(\mathbf{g}) + \varphi(\mathbf{h} - \mathbf{g}) + \varphi(-\mathbf{h}), \tag{1}$$

well known from direct methods. This is a combination of three structure-factor phases, the corresponding reciprocal-lattice vectors of which join to form a closed polygon. Here, $\mathbf{h}$ denotes the reciprocal-lattice vector (r.l.v.) of the primary reflection, the intensity of which will be modulated owing to excitation of the secondary r.l.v. $\mathbf{g}$ through coupling *via* $\mathbf{h} - \mathbf{g}$. Single phases can be obtained only if two of the reflections participating in a three-beam case are related by symmetry in such a way that their phases cancel, as in a Σ_1 relation (Giacovazzo, 1980), *e.g.*

$$\Phi_T = \varphi(\mathbf{g}) + \varphi(\mathbf{gR}) + \varphi(-\mathbf{h}_s) = \varphi(-\mathbf{h}_s) + 2\pi\mathbf{gt}. \tag{2}$$

Here $(\mathbf{R}, \mathbf{t})$ are the rotational and translational parts of a symmetry operation of the corresponding space group and $\mathbf{h}_s$ represents a semi-invariant reflection.

The structure-factor moduli of the reflections participating in a three-beam case have to remain in a certain relation to each other in order to avoid large contributions of phase-independent effects such as *Umweganregung* and *Aufhellung*. For the three-beam interference experiments presented below with protein crystals, the rough rule of thumb

$$3 < [|F'(\mathbf{g})||F'(\mathbf{h} - \mathbf{g})|]/|F'(\mathbf{h})|^2 < 8 \qquad (3)$$

was applied. F' denotes the structure-factor modulus F corrected for polarization.

In theory, the range given in (3) for the determination of a triplet phase from three-beam interference profiles would be larger. However, in order to keep the pure interference effect (which depends on the triplet phase) large compared with phase-independent effects (which are independent of the sign of the triplet phase) and taking into account experimental errors arising from counting statistics, crystal quality (mosaicity) and crystal-shape effects (Weckert & Hümmer, 1998), a criterion similar to equation (3) has to be applied in order to obtain reliable phase information. Owing to the dense reciprocal lattice of a protein crystal, the interference profiles of many three-beam cases will overlap (Weckert & Hümmer, 1997). Therefore, the only three-beam cases which are useful are those which give rise to a significantly larger interference effect compared with all neighbouring cases. In general, the overlap of three-beam cases with large interference effects can be avoided by choosing a suitable wavelength. For the measurements reported here, the following condition was fulfilled for the measured three-beam case $\mathbf{h}/\mathbf{g}/\mathbf{h} - \mathbf{g}$ and its neighbours $\mathbf{h}/\mathbf{g}'/\mathbf{h} - \mathbf{g}'$:

$$q = [|F(\mathbf{g}')F(\mathbf{h} - \mathbf{g}')|]/[|F(\mathbf{g})F(\mathbf{h} - \mathbf{g})|] < 0.25. \qquad (4)$$
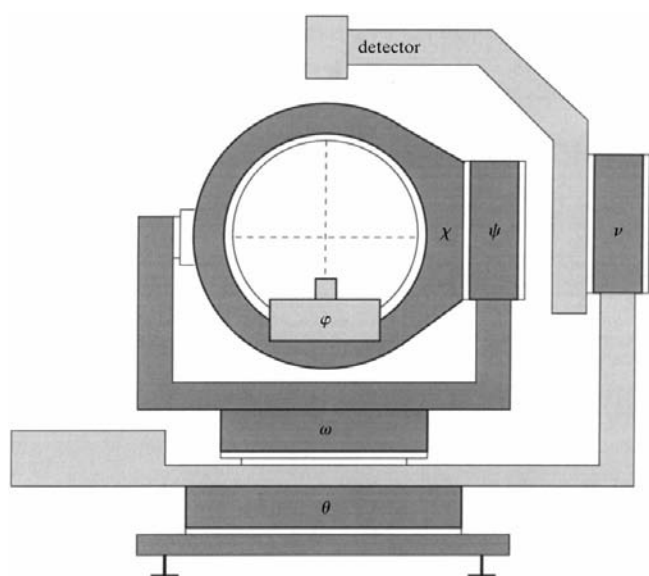

Figure 1
Ψ-circle diffractometer: the axes ψ and χ are used to align the primary r.l.v. $\mathbf{h}$ with the ψ axis. Axis ω is used to adjust the Bragg angle. Azimuthal rotation about $\mathbf{h}$ can be accomplished by the ψ axis.

As a result of the combination of equations (3) and (4), only three-beam cases with reflections having the largest structure-factor moduli give rise to interpretable interference effects. For the case of tetragonal lysozyme, this corresponds to about 3000 reflections with the largest structure-factor moduli.

2. Experimental

2.1. Instrumentation

It has already been mentioned that a tunable source allows one to avoid the overlap of significant three-beam interferences. Synchrotron radiation is clearly required for this kind of experiment, especially if the measurement of a large number of triplet phases is envisaged. Since the interference effects are more distinct using a parallel incident beam, focusing may provide only a minor advantage. In most cases, the crystals are also quite small. Therefore, the brilliance of the synchrotron is more important than the flux.

The triplet-phase information is contained in the profile form $I(\Psi)$ of the interference effect. The angle Ψ represents the azimuthal rotation about the r.l.v. $\mathbf{h}$. Therefore, the primary beam must have a homogeneous angular intensity distribution. Otherwise, the intensity distribution of the primary beam will spoil the interference effects.

The triplet phases were collected at room temperature using synchrotron radiation from a bending magnet either at DORIS (HASYLAB, Hamburg, Germany) or at the ESRF (Swiss–Norwegian beamline, Grenoble, France). A fixed-exit double-crystal Si(111) monochromator was used, operating in vertical mode and giving a bandwidth of approximately 0.01–0.03%. The wavelengths used for the three-beam experiments covered the range 0.9–1.3 Å. The divergence is approximately given by the size of the source (electron beam) plus that of the crystal, divided by the distance between them. With a crystal size of 0.3 mm, the divergence at DORIS is about 0.002° in the vertical direction and 0.006° in the horizontal direction, whilst at the ESRF bending magnet it is about 0.0007° in both directions.

During a Ψ-scan experiment the direction of the primary reciprocal-lattice vector $\mathbf{h}$ must not change relative to the Ewald sphere (direction of the incoming beam). To accomplish this, a special high-resolution Ψ-circle diffractometer (Fig. 1) has been constructed which is able to carry out a Ψ-scan by turning about a single axis only (Weckert & Hümmer, 1997).

2.2. Crystals

Before the measurement of three-beam interference profiles of a given crystal, suitable conditions (wavelength, setting mode and Ψ angle) for the three-beam case have to be selected. The following data are needed: (i) the metric parameters of the unit cell, (ii) the orientation parameters for the individual crystal and (iii) an F_{obs} data set which is as complete as possible, including all low-resolution reflections. The most critical factor for this kind of experiment is crystal quality, since interference effects can take place only within a perfect

mosaic block. Protein crystals in general are not perfect. Therefore, interpretable three-beam effects can be obtained only for crystals where either (i) the alignment of the individual blocks is sufficiently close that the corresponding interference effects overlap within the experimental resolution (*e.g.* Fig. 2) or (ii) the single blocks are well separated so that they can be selected individually by the parallel synchrotron-radiation beam (*e.g.* Figs. 3 and 4). Many protein crystals showed a rather small mosaic spread, of the order of 0.01°, and often showed a mosaic spread of about 0.002° at the limit of the resolution of the experiment. However, crystals with a sm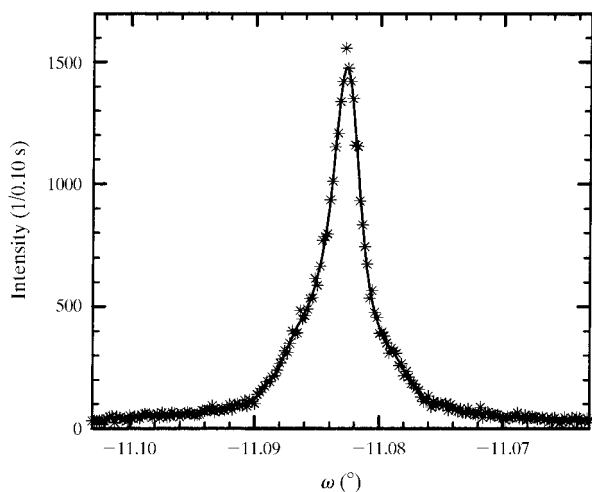ooth orientation distribution of small mosaic blocks in general give no interference profiles which could be interpreted.

2.3. Measurement procedure

The measurement procedure has already been described in detail elsewhere (Weckert & Hümmer, 1997). After adjustment to a suitable wavelength, the primary r.l.v. $\mathbf{h}$ is aligned accurately with the ψ axis of the diffractometer and the rocking curve of the primary reflection $\mathbf{h}$ is measured by an ω scan. The crystal is then positioned at the angular position of the maximum of the intensity profile derived from this measurement. For narrow reflection profiles ($\simeq 0.002°$) this task is not trivial because of the stick-and-slip behaviour of the mechanics. In the next step, the reflection profile of the secondary reflection $\mathbf{g}$ is measured by an azimuthal Ψ scan about $\mathbf{h}$. The position of the maximum of this rocking curve serves as a reference point for the Ψ-scan three-beam interference experiment. The latter scan requires step widths of 0.0002–0.001°, depending on the mosaicity of the crystals. In order to average short time instabilities, the final profile consists of a number of single scans with different step-scan times, *e.g.* $(0.05 + R \times 0.05)$ s for each scan. R represents a random number between 0 and 1 which changes between individual scans. The experiment is interrupted as soon as the counting statistics are sufficient for an assignment of a triplet phase. For each triplet-phase determination, two interference profiles have to be recorded, $\mathbf{h}/\mathbf{g}/\mathbf{h} - \mathbf{g}$ and $-\mathbf{h}/-\mathbf{g}/\mathbf{g} - \mathbf{h}$. This measurement strategy enables one (i) to evaluate *Umweganregung* and *Aufhellung* contributions (Weckert & Hümmer, 1990; Hümmer *et al.*, 1990) and (ii) to carry out a consistency check, which is important for poor-quality crystals. Depending on the crystal quality and the size of the interference effect, the total measurement time for the determination of a triplet phase is about 10–15 min at an ESRF bending-magnet beamline.

Figure 2
Rocking curve of the $(00\overline{36})$ reflection (FWHM: 0.0034°) from a proteinase K crystal at $\lambda = 1.1572$ Å at an ESRF bending-magnet beamline. The influence of some smaller mosaic blocks can be seen close to the tails of the profile. The solid line shown in all experimental profiles represents the most probable smooth curve to the data points calculated by a generalized cross-validation algorithm (Craven & Wahba, 1979).
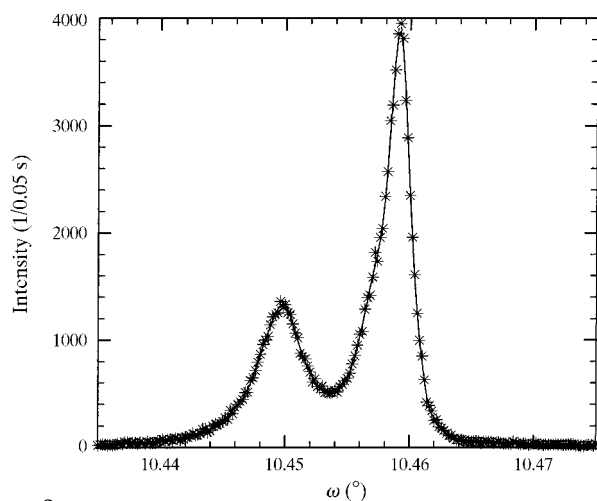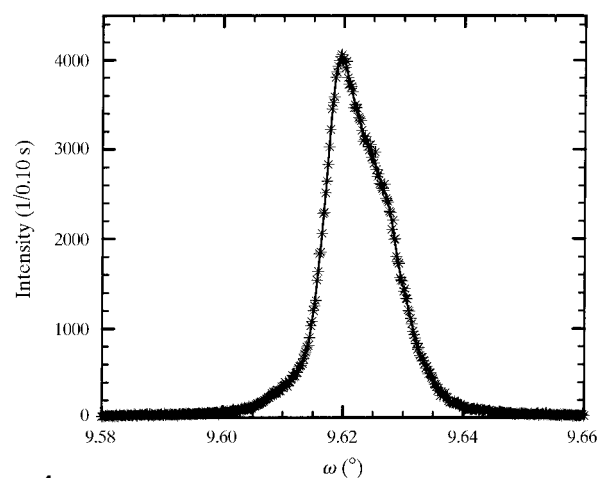

Figure 3
Rocking curve of the $(5\,\overline{13}\,\overline{8})$ reflection (FWHM = 0.0028°) from a crystal of tetragonal lysozyme at $\lambda = 1.3222$ Å. This crystal consisted of at least two blocks which can easily be separated by the parallel synchrotron beam of an ESRF bending magnet.

Figure 4
Rocking curve of the $(\overline{7}45)$ reflection (FWHM = 0.0112°) from a triclinic lysozyme crystal at $\lambda = 1.2454$ Å. The shoulder on the right side of the profile indicates a second mosaic block. Even in a case like this, the beam is sufficiently parallel to select only the main block for the three-beam interference experiment (see Fig. 11).

3. Results

Hen egg-white lysozyme was selected for systematic investigation since the compound is cheap and good-quality crystals of both the tetragonal and the triclinic modifications can be grown. The water content of the triclinic modification is much less than that of the tetragonal modification. The triclinic modification also shows significantly smaller atomic thermal displacement parameters and a less dense reciprocal lattice. For these reasons, the triclinic modification was chosen to find the resolution limit to which interference effects can be observed for molecules of this size. In addition, the tetragonal modification served as a test structure for the development of a structure-solution method based on experimentally determined triplet phases in connection with statistical methods (Weckert, 1999). Furthermore, in additional experiments the feasibility and speed of the method were tested with a larger protein structure (proteinase K).

3.1. Tetragonal lysozyme

Tetragonal lysozyme crystallizes in space group $P4_32_12$, with unit-cell parameters $a = 79.1$, $c = 37.9$ Å [PDB (Bernstein *et al.*, 1977) entry 1lyz; Diamond, 1974]. It is a small protein containing 129 amino-acid residues, a total of 1001 non-H atoms. The molecular weight is about 14.6 kDa. The sizes of the various crystals investigated ranged from $0.7 \times 0.3 \times 0.2$ mm to $1.5 \times 1.0 \times 0.9$ mm.

3.1.1. Intensity-data collection. As mentioned in §2.2, a complete intensity-data set, including low-resolution reflections, is required for estimation of the interference effect of the main and neighbouring three-beam cases. Therefore, data sets on different crystals, from very low resolution (using a high-resolution diffractometer) to medium resolution (using an imaging-plate scanner) were collected with radiation from a rotating-anode X-ray generator. These data sets were merged with a high-resolution data set (14.7–1.34 Å) (PDB entry 193l; Vaney *et al.*, 1996) to form a nearly complete data set, which we used for the selection of three-beam cases to be measured and to estimate the influence of neighbouring three-beam cases.

3.1.2. 'Triplet-phase' data collection. A total of 847 triplet phases were collected from 23 different crystals. Among them were 72 triplet phases measured at beamline C at HASYLAB (DESY, Hamburg). All others were collected at the Swiss–Norwegian beamline at the ESRF (Grenoble). During the first stages of the development of this method, the number of triplet phases which could be obtained from one crystal was quite small ($\simeq 10$). After optimization of the measurement protocol and by moving the experimental setup to the ESRF, it was possible to determine between 80 and 150 triplet phases from a single crystal.

The limiting factor for the number of triplet phases from one crystal is the influence of the radiation damage. For example, crystals of lysozyme can withstand an unfocused beam from an ESRF bending magnet for 24–36 h. During that time, the intensity change owing to the interference effect decreases by 50% (Weckert & Hümmer, 1997). Therefore, we

monitor the crystal quality by occasional remeasurement of a standard three-beam case during a triplet-phase data collection. This approach is similar to the repeated measurement of standard reflections in a normal diffractometer intensity-data collection. Since three-beam interference profiles are much more sensitive to crystal quality than rocking curves, the monitoring of the state of the crystal's decay cannot in general be accomplished by a simple rocking-curve measurement.

In order to reduce radiation damage, an attempt was made to apply the cryo-techniques which are almost standard for intensity-data collection (Garman & Schneider, 1997). For successfully frozen crystals, an increase in the mosaic spread (0.15–0.5°) was observed which was too large to allow three-beam interference experiments, but still small enough for routine intensity-data collections. These results will be published elsewhere (Weckert, 1999).

The time required for one triplet-phase determination depends on the primary beam intensity, crystal quality and size and on the magnitude of the interference effect of the particular three-beam case. For each triplet phase, two interference profiles were measured (Weckert & Hümmer, 1997). Each of these measurements includes two rocking curves in order to determine accurately the positions of the primary and secondary reflections and subsequently the interference profile itself. On a good-quality lysozyme crystal with a size of about $0.7 \times 0.5 \times 0.5$ mm at a bending magnet of the ESRF, it was possible to determine as many as six triplet phases within 1 h when the d-spacing of the reflection to be phased was in the range 3–6 Å. Therefore, six triplet phases correspond to 36 measured profiles. The size of the interference effect is reduced by crystals of worse quality and also by larger protein structures; the time required for each three-beam profile will increase in these cases. The distribution of the relative intensity changes arising from these interference effects in tetragonal lysozyme is shown in Fig. 5. From all measured three-
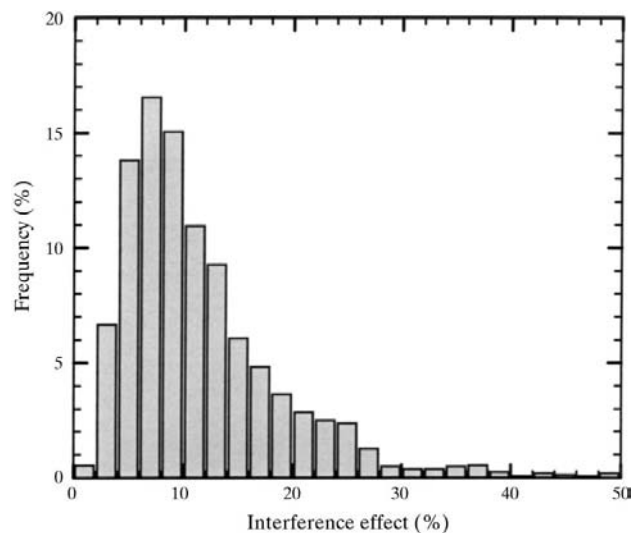

Figure 5
Distribution of the relative intensity change owing to the interference effects of the measured three-beam interference profiles from tetragonal lysozyme.

beam cases, 87% have an intensity change less than 20%; 40% showed an intensity change between 5 and 10%. An example for a three-beam interference profile of tetragonal lysozyme is given in Fig. 6. Despite the fact that this crystal consisted of more than one block, the parallel synchrotron beam allowed us to pick the largest one for the interference experiment. In addition to the main three-beam case, the small influence of neighbouring three-beam cases is visible.

Visual interpretation of the interference profiles from a single three-beam case and its centrosymmetrically related case allows the estimation of triplet phases in steps of about 15° (Weckert & Hümmer, 1997). In addition, an automatic empirical fitting procedure was applied to reduce possible interpretation errors (Hölzer, 1998). To check the reproducibility of the experimental determination of triplet phases, a selection of three-beam cases were measured on different lysozyme crystals. These twice-determined triplet phases agreed within 15°.
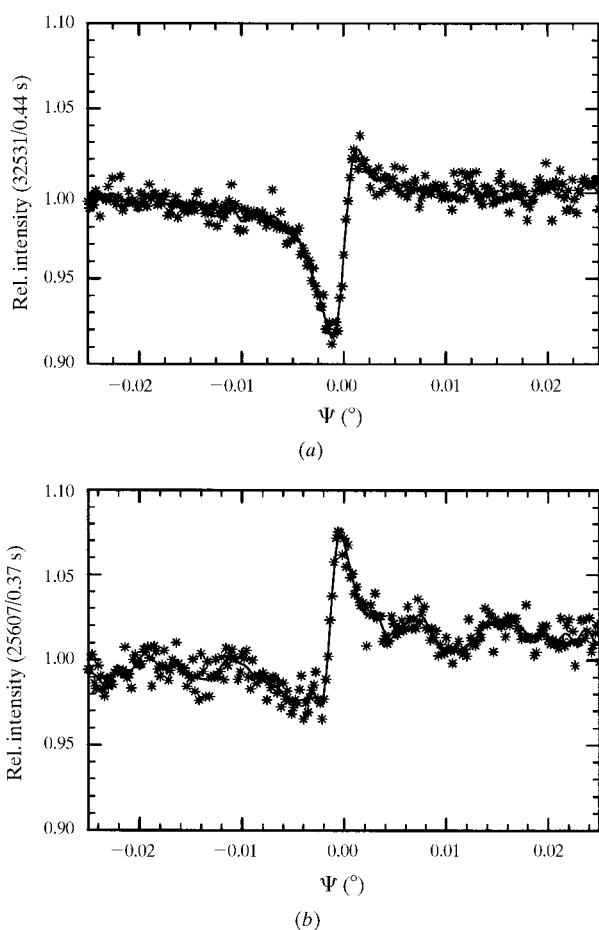
The mean phase difference of all measured triplet phases compared with a recent model of the known structure is 17.5° (PDB entry 193l; Vaney *et al.*, 1996). Occasionally, differences up to 180° were observed between the estimated and the calculated triplet phases, especially where reflections with small structure-factor moduli are involved. It has to be mentioned that *calculated* triplet phases for two different PDB entries describing the same structure can in some cases also show differences up to 180°. These differences in the phases of reflections with smaller structure factors might result from slight non-isomorphism between different crystals (Vaney *et al.*, 1996 and references therein). The distribution of the triplet-phase differences to PDB entry 193l is shown in Fig. 7. A list of all measured triplet phases has been deposited.[1]

3.1.3. Derived structure-factor phases. During any structure-solution process, single phases are necessary, *e.g.* for the calculation of a map. The experimentally determined triplet phases contain 752 individual reflections. Among them are 26 triplet phases which are Σ_1 relationships, from which a single semi-invariant phase can be deduced. Using these phases, and after selecting two origin-fixing reflections, the measured triplet phases can be connected to form a phasing tree to provide single phases. The phases of those reflections which cannot be assigned a reliable value can be determined by statistical methods. This can be accomplished by assigning them symbolic phases which will be permuted using, for example, the magic integer algorithm (Main, 1977). This gives a number of phase sets which have to be ranked by a suitable figure of merit. It has been found that a maximum-entropy based approach using likelihood ranking (Bricogne, 1984; Bricogne & Gilmore, 1990) can be used to solve this problem. Further results regarding this technique will be published elsewhere (Weckert, 1999). In comparison with the known model (PDB entry 193l) a mean weighted structure-factor phase difference of about 16° has been obtained for the most likely solution of the maximum-entropy procedure. This value is slightly smaller than the phase difference for triplet phases, since a number of single phases are determined by more than one measurement and therefore measurement errors are averaged. In Figs. 8 and 9, the completeness of the phased reflections is shown as function of resolution and structure-factor modulus, respectively. The *d*-spacing ($1/|\mathbf{h}|$) of the reflections involved in the three-beam cases extends from 55.5 to 2.5 Å, with the maximum of the distribution at 4 Å. The structure-factor modulus of the reflections involved in the three-beam cases extends from 200 to 1500, with the maximum of the distribution at 600. Since it is hardly possible nor necessary to measure the phases of all structure factors, we attempted to phase reflections equally distributed over all directions in reciprocal space. In Fig. 10, one-eighth of the stereographic projection of all phased reflections including symmetry equivalents is shown. These 752 phased reflections carry sufficient structural

Figure 6
Interference profiles of (*a*) the three-beam case $\bar{5}\,1\,3\,8/1\,2\,0/\bar{6}\,1\,1\,8$ and (*b*) the centrosymmetrically related three-beam case $5\,1\,\bar{3}\,\bar{8}/\bar{1}\,\bar{2}\,0/6\,\bar{1}\,\bar{1}\,\bar{8}$ from a crystal of tetragonal lysozyme at $\lambda = 1.3222$ Å with an estimated triplet phase of (*a*) $\varphi_T = 135°$ and (*b*) $\varphi_T = -135°$. The calculated triplet phase is $\varphi_T^{193l} = \pm 140°$ (PDB entry 193l). The rocking curve for this crystal is shown in Fig. 3. The secondary r.l.v. $\mathbf{g}$ is inside the Ewald sphere for negative values of ψ. For all three-beam interference profiles, the mean intensity and total measurement time per data point are given.

information to give an interpretable electron-density map (Hölzer *et al.*, 1997).

3.2. Triclinic lysozyme

Triclinic lysozyme crystallizes in the space group *P*1, with unit-cell parameters *a* = 27.283, *b* = 31.980, *c* = 34.291 Å, α = 88.53, β = 108.57, γ = 111.85° (PDB entry 2lzt; Hodsdon *et al.*, 1990). The molecular weight is 14.6 kDa. The sizes of the various crystals investigated were between 0.3 and 0.5 mm. During these test experiments 47 triplet phases were determined. Among them are 13 triplet phases measured at beamline C at HASYLAB (DESY, Hamburg). All others were measured at the Swiss–Norwegian beamline at the ESRF (Grenoble). The mean triplet-phase difference between the experimentally determined phases and the phases calculated

from the PDB entry 2lzt is 21°. The maximum of the distribution of the phased reflections over the resolution range is at 4 Å. A list of the measured triplet phases has been deposited.[1]

Even when reflections with a resolution as high as 2.0 Å were involved in three-beam cases, it was still possible to observe interference effects of about 10% intensity (Weckert & Hümmer, 1997). This must be compared with the maximal achieved resolution of about 2.5 Å for interpretable three-beam measurements with the tetragonal modification. This higher resolution for the triclinic form was in spite of the fact that, in general, the triclinic modification showed a slightly
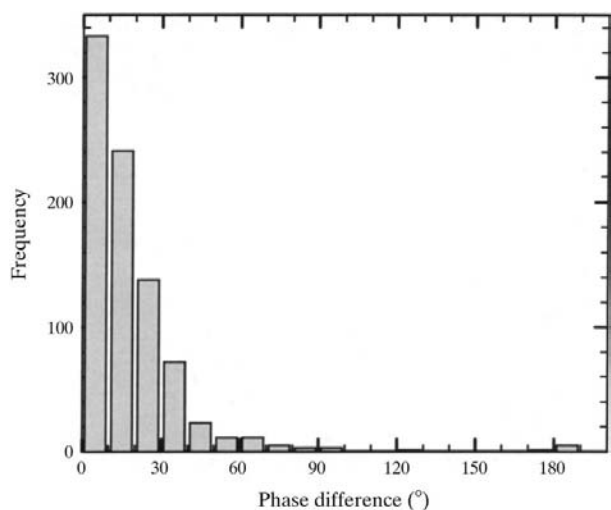

Figure 9
Completeness of phased reflections of tetragonal lysozyme as a function of structure-factor modulus.

Figure 7
Distribution of the phase difference of the measured triplet phases from tetragonal lysozyme compared with calculated values from PDB entry 193l.
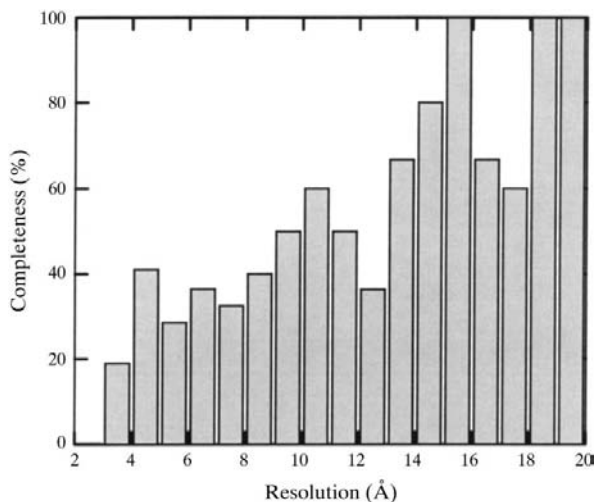

Figure 8
Completeness of phased reflections of tetragonal lysozyme as a function of resolution.
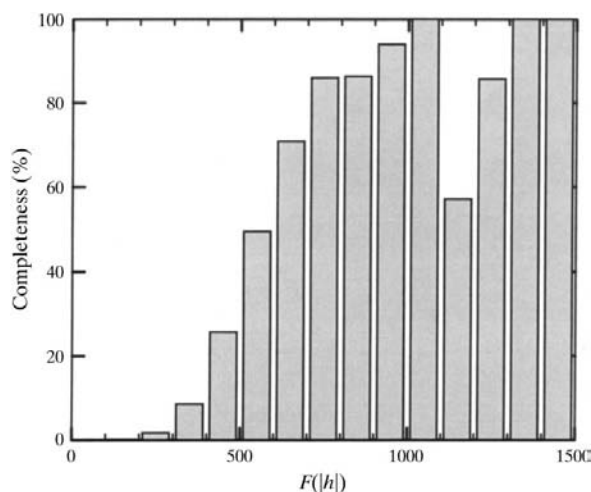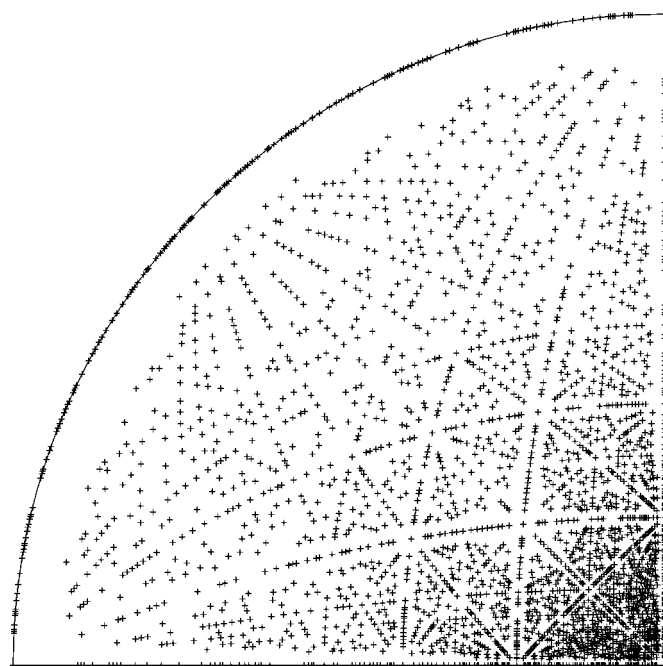

Figure 10
One-eighth of the stereographic projection of the directions of the reciprocal-lattice vectors from all phased reflections of tetragonal lysozyme, including symmetry equivalents.

higher mosaic spread. Likely reasons for this behaviour are the smaller atomic thermal parameters in the triclinic crystal and a less dense reciprocal lattice because of the more than eightfold smaller unit cell.

An example for a three-beam interference profile of the triclinic modification is shown in Fig. 11. Besides the three-beam case $7\bar{4}\bar{5}/0\bar{1}1/7\bar{3}\bar{6}$, two neighbouring three-beam cases $\mathbf{h}/\mathbf{g}'/\mathbf{h} - \mathbf{g}'$ with the primary reflection $7\bar{4}\bar{5}$ can also be observed. These neighbouring three-beam cases are not optimized for this wavelength and are, therefore, severely influenced by other small-effect neighbours. Hence, no reliable phase information can be retrieved from interference profiles for which the wavelength has not been optimized with respect to the influence of other smaller neighbouring three-beam cases.
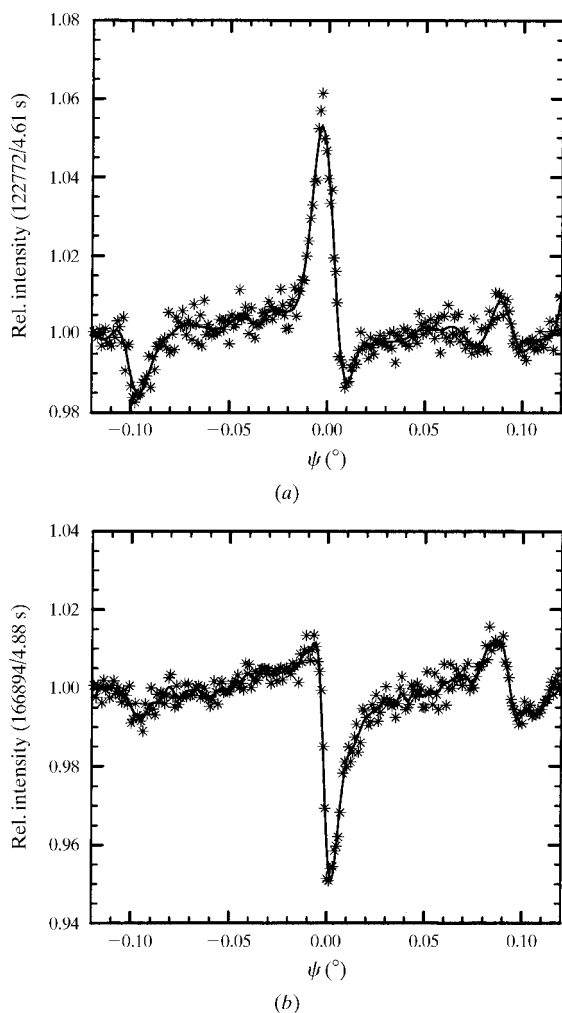

(a)

(b)

Figure 11
Interference profiles of (*a*) the three-beam case $7\bar{4}\bar{5}/0\bar{1}1/7\bar{3}\bar{6}$ and (*b*) the centrosymmetrically related three-beam case $\bar{7}45/01\bar{1}/\bar{7}36$ from a triclinic lysozyme crystal at λ = 1.2454 Å with an estimated triplet phase of $\varphi_T = -60°$ for (*a*) and $\varphi_T = 60°$ for (*b*). The calculated triplet phase is $\varphi_T^{2lzt} = \mp 52°$. The ψ-rotation sense is the same as described in Fig. 6. The second mosaic block visible in Fig. 4 has no influence on the interference profile, since it is not fulfilling the diffraction condition for the narrow incoming beam.

3.3. Proteinase K

Proteinase K crystallizes in space group $P4_32_12$ with unit-cell dimensions a = 68.2, c = 108.3 Å (PDB entry 2prk; Betzel *et al.*, 1988). This protein contains 279 amino-acid residues, a total of 2020 non-H atoms. The molecular weight is about 28.8 kDa. The size of the crystal used in the measurements was about $0.2 \times 0.2 \times 0.1$ mm.

Proteinase K was chosen to investigate proteins with larger unit cells (5×10^5 Å^3). This is about twice the volume of tetragonal lysozyme. As the molecular size increases, the mean intensity of the reflections decreases relative to the total scattering power of the unit cell. Also, as the size of the unit cell increases, the density of reflections in reciprocal space increases. Therefore, it is more difficult to find suitable three-beam cases free from the influence of strong neighbouring cases [see (3) and (4)].

It was possible to obtain high-quality crystals of proteinase K (see Fig. 2). Despite the small crystals and the large unit cell, it was possible to determine three triplet phases per hour with at least one of the reflections involved in the 3–6 Å resolution range. The mean phase difference relative to the known model was in the same order of magnitude as previous experiments with lysozyme.

In Fig. 12, an example of the interference profile of proteinase K is shown. This is an example of a four-beam case where the interference profiles of two symmetry-equivalent three-beam cases superpose (Huang *et al.*, 1994). Thus, the interference effect is slightly larger than from a single three-beam case alone. The influence of quartet terms also present in a four-beam case is of higher order (Hümmer, Bondza *et al.*, 1991) and cannot be observed in this profile.

4. Discussion and conclusions

In the previous sections, the details of three-beam interference experiments on different proteins are described. To demon-
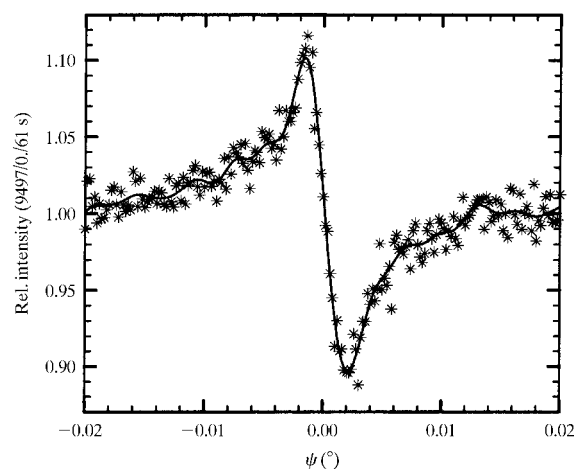

Figure 12
Interference profile of the two symmetrically related three-beam cases $00\bar{3}6/11\bar{2}/\bar{1}\bar{1}34$ and $00\bar{3}6/11\bar{3}4/\bar{1}\bar{1}2$ from a proteinase K crystal at λ = 1.1572 Å with estimated triplet phase $\varphi_T = 0°$ and a calculated triplet phase of $\varphi_T^{2prk} = 0°$. The ψ-rotation sense is the same as described in Fig. 6.

strate an *ab initio* structure solution based on experimentally determined triplet phases, a large set of three-beam interference profiles was collected from HEW lysozyme. In order to calculate an electron-density map, structure-factor phases (single phases) can be derived by building up a phasing tree from the experimentally determined triplet phases. Only a small number of these phases could not be assigned and had to be determined by using a statistical approach. In the case of an unknown protein structure, this number will certainly be higher, giving more importance to the statistical part of this approach.

Since the phase error is comparatively small and the phased reflections are those with the largest structure-factor moduli, quite a small number of phases are needed to calculate an interpretable electron-density map compared with the total number of reflections in a high-resolution intensity-data set. For lysozyme, 750 reflections proved to be more than enough (Hölzer, 1998; Hölzer *et al.*, 1998). It is expected that more elaborate methods of using experimental triplet phases might require an even smaller number.

The number of measured phases per hour is low compared with a modern intensity-data collection using an area-detector system. Some increase in the rate has been achieved by using radiation from an ESRF undulator beamline. There are, however, limitations such as the maximum driving speed of a diffractometer and the maximum flux that an uncooled protein sample can withstand for a reasonable amount of time.

Some restrictions of the method have already been discussed above. Only the phases of reflections with large structure-factor modulus can be obtained. This seems to be no real restriction. Since flash-freezing cannot be applied, the crystals must be reasonably stable in the beam. Theoretically, there is no lower limit in size for the occurrence of three-beam interference effects, *e.g.* they can also be observed in a few nanometre thick epitaxic layers (Schroer, 1998). In practice, however, it is extremely difficult to measure triplet phases from crystals of size smaller than 80–100 µm, since the number of incident and scattered photons per crystal volume is too high to obtain sufficient statistics for the measurement. This leads to rapid radiation decay. Another restriction is the possible size of the unit cell. Experimental evidence for three-beam interference effects has been obtained for structures up to a unit-cell size of 1.2×10^6 Å^3 (Weckert *et al.*, 1993). At present, we have no experience with crystals having a larger unit cell.

The main disadvantage of the method is the requirement for good-quality crystals. However, finding suitable crystals for the proteins presented in this communication was not difficult. In addition, there are also other proteins (Mo *et al.*, 1998) for which three-beam interference profiles have been observed. The feasibility of this kind of experiment depends strongly on the nature of the mosaic spread, as discussed above. The only way to determine whether a crystal can be used for three-beam experiments is a high-resolution rocking-curve measurement. If the rocking-curve width of a single mosaic *block* of a protein crystal is larger than 0.02°, then the size of

three-beam interference effects becomes quite small. Unfortunately, the values of 'mosaic spread' produced by intensity-integration software are only a rough guideline depending on the experimental conditions. If the 'mosaic spread' observed is close to the values which are characteristic for a certain beamline setup for well scattering crystals, then it is probably worth trying a three-beam interference experiment. If it is higher, it will be very unlikely that three-beam interferences can be measured.

A clear advantage of the three-beam interference method is that the crystals do not have to be modified at all. Triplet phases can be obtained independently of the chemistry of the crystal, provided the energy range is not too close to an absorption edge. In the measurement technique we have devised, *i.e.* comparing the profiles of two centrosymmetrically related three-beam cases, the phase error is small compared with most other methods. Considering the experimental effort necessary for the three-beam interference method, it is certainly only warranted for structures which cannot be solved easily by other more standard methods and where suitable crystals are available. When high-quality and stable native crystals are available, three-beam phasing might lead to a structure solution more quickly than some other methods.

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