Direct Phase Determination of Large Macromolecular Crystals Using Three-Beam X-Ray Interference

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We report for the first time the direct determination of x-ray-reflection phases of a large-unit-cell volume, 350000 Å^3, macromolecular crystal using three-beam diffraction. The crystal polarity (enantiomorph) is determined unambiguously from the peak-intensity measurement. Quantitative analysis of phase angle, with a scaling scheme for intensities, is presented.

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Phase, a relative physical quantity, appears frequently as an important parameter in many physical systems. In the case of x-ray diffraction from single crystals, the phases of structure factors play a decisive role in determining the relative positions of atoms in the crystal unit cell. However, the phase information is lost in the diffraction intensity measurement, because the intensity is only related to the product of the structure factor and its complex conjugate. This fact constitutes the well-known x-ray phase problem in diffraction physics and crystallography.

X-ray multiple diffraction, which involves coherent interaction (interference) among the diffracted waves inside a crystal, has long been considered as a possible means of solving this phase problem [1]. Recent progress [2-9] in utilizing three-beam diffraction techniques for phasing has revealed the capabilities of determining the sign [4] and magnitude [6,7] of cosδ, and of providing semiquantitative [8] and quantitative [9] information about the triplet phase δ. These efforts have led to the phase determination of reflections from organic crystals [7,10,11], though the quantitative approach [9] is limited to weak reflections due to extinction effects. In addition, using elliptically polarized x rays as an alternative way of determining the sign of sinδ has also been carried out for perfect GaAs crystals [12]. Very recently a semiquantitative phase determination of a small macromolecular crystal, 66854 Å^3 in unit-cell volume, has been demonstrated [13].

Often the multiwavelength anomalous scattering and the isomorphous replacement methods, among others, are employed to determine the phases for macromolecular crystals [14]. However, in some cases, the necessary heavy-atom derivatives or a proper spectral range of radiation are not attainable and an alternative method of phasing process is needed. As a first step towards this challenging goal, we apply the multiple diffraction technique to extract phase information from the horse *oxy*hemoglobin single crystals of known structure [15] (monoclinic, space group $C2$, cell dimension: $a=108.24$ Å, $b=63.13$ Å, $c=54.54$ Å, $\beta=110.85°$, $V=348277$ Å^3). In this Letter, we demonstrate that the phases can be determined by measuring the intensities of three-beam diffraction from this large macromolecular crystal. A scaling scheme employed in the quantitative phase analysis is also reported.

Multiple diffraction takes place when a crystal is first aligned for a Bragg reflection G and then rotated (ψ scan) around the reciprocal lattice vector $\mathbf{g}$ of the G reflection to bring additional sets of atomic planes, say, L planes, to satisfy simultaneously Bragg's law. In a three-beam case, denoted by G/L, there are three reflections involved: the primary G, the secondary L, and the coupling reflection G-L. The interaction among the diffracted beams gives rise to intensity variation on the primary beam. This variation is related to the phase δ of the structure-factor triplet $F_L F_{G\text{-}L}/F_G$ [6].

Because of the large-crystal unit cell, the number of multiple diffraction conditions in a 1° ψ scan is estimated to be about 8900 diffractions for $\lambda=1.54$ Å, 1400 for $\lambda=1.8$ Å, and 1000 for $\lambda=2.0$ Å. With such densely spaced diffractions in a small angular range, observable diffraction peaks can be obtained only for strong diffractions. For this reason we calculate the intensity of each multiple diffraction according to the kinematical theory [16]. The Lorentz polarization factor is included to take care of the peak width and beam polarization. The lattice parameters, wavelength, and the intensities of Bragg (two-beam) reflections from the protein data bank [17] are used as the input for this calculation. From these considerations we choose, among 30 strong diffractions, the three-beam cases, $5\bar{1}\bar{2}/60\bar{2}$ and $\bar{5}12/\bar{6}02$, with $\{5\bar{1}\bar{2}\}$ as the primary reflection for investigation (here we combine the ideas of Refs. [8,9] to use a pair of centrosymmetrically related diffractions for later quantitative phase analysis). The suitable wavelength is around 2 Å. The fact that the intensities of the chosen three-beam diffractions are at least 2 orders of magnitude stronger than those of the diffractions nearby ensures the visibility of well-resolved diffraction peaks above the two-beam background.

The multibeam experiments were performed on beam line X10A at the National Synchrotron Light Source, Brookhaven National Laboratory. The incident x rays from the synchrotron source were focused with a bent cylindrical mirror and monochromatized with a pair of Ge(111) crystals. Two pairs of slits, vertical and horizontal, in front of the sample crystal were used to lower the beam divergence to 0.01° in both directions. A scintillation counter placed behind the two sets of slits acted as a monitor for intensity normalization. A Huber four-circle diffractometer was used. A ruby crystal was employed to double-check the diffractometer alignment and to calibrate the wavelength ($\lambda = 2.0872$ Å). The diffracted beam was monitored by a second scintillation counter with a single x-y slit in front of it. Evacuated beam paths were used to reduce the air scattering. The distances from the two pairs of collimation slits to the sample crystal were 77 and 26 cm, respectively, while the distance from the receiving slit to the crystal was 33 cm.

The hemoglobin crystal, $0.5\times0.5\times0.2$ mm^3, was mounted on the diffractometer, and utilizing the diffraction spots from a 360° rotation photograph, the orientation matrix of the crystal was determined. The $5\bar{1}\bar{2}$ planes were then set into diffraction position, and the incoming and outgoing positions for a three-beam diffraction were calculated (at these two positions the reciprocal lattice point L of the secondary reflection moves across the surface of the Ewald sphere) [18]. A position, which can be reached by the combined ω, ϕ, and χ rotation of the diffractometer, was chosen to perform the ψ scan for the three-beam case.

Since the ψ scan is a combined ω, ϕ, and χ rotation, slight angle-missetting errors can cause undesirable peak-intensity variations; therefore, we performed ω scans (rocking) at each ψ position to track the peak position so the intensity variation due to three-beam interaction could be detectable. Figure 1(a) shows the rocking curves at the peak (solid curve) and off the peak (dashed curve) for the three-beam diffraction $5\bar{1}\bar{2}/60\bar{2}$ in the outgoing (OUT) situation (the dashed curves were repeatedly observed before and after the three-beam position). The intensity enhancement at the peak is seen. Deterioration of the crystal under radiation was another problem encountered. Figure 1(b) shows the rocking curves for the $\bar{5}12/\bar{6}02$ (IN) case. The presence of two large mosaic blocks is obvious and the third small mosaic is also observed. These scans were taken about 2 h after those shown in Fig. 1(a). Though the crystal gradually decayed, the intensity enhancement of the aligned mosaic was still detectable. The integrated intensities over ω vs $\Delta\psi$ during the ψ scans, 0.005° per step, are plotted in Fig. 2 for the $\bar{5}12/\bar{6}02$ and $5\bar{1}\bar{2}/60\bar{2}$ cases. $\Delta\psi$ is the angular deviation from the exact three-beam position. The intensities were normalized according to the integrated two-beam intensity of $\{5\bar{1}\bar{2}\}$ for a single mosaic block after background subtraction. The curve of $5\bar{1}\bar{2}/60\bar{2}$ was inverted about $\Delta\psi = 0$ so that the two profiles can be compared and analyzed in the same incoming situation.

According to Ref. [9], the relative intensity distribution I'_G of a three-beam case consists of the phase-dependent part I_D and phase-independent part I_K [19]:

$$I'_G(\Delta\psi) = [I_G(3) - I_G(2)]/I_G(2) = I_D + I_K\,, \tag{1}$$

where $I_G(2)$ and $I_G(3)$ are the two- and three-beam intensities of the primary reflection. I_K is a symmetric function of $\Delta\psi$. I_D is defined as

$$I_D = A[2(\Delta\psi)\cos\delta - \eta\sin\delta]\,, \tag{2}$$

where A depends on the structure-factor moduli, Lorentz polarization factor, and $\Delta\psi$ [9]. η is the full width at half maxima (FWHM). At the peak position ($\Delta\psi = 0$) and

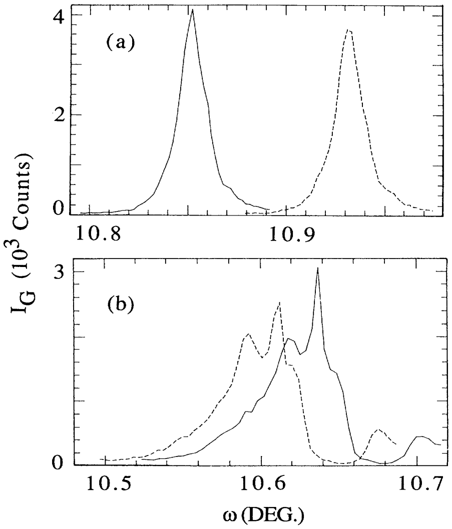

FIG. 1. Rocking curves of the primary (a) $5\bar{1}\bar{2}$ and (b) $\bar{5}12$ reflections at (solid curve) and off (dashed curve) the three-beam $5\bar{1}\bar{2}/60\bar{2}$ (OUT) and $\bar{5}12/\bar{6}02$ (IN) positions, respectively.

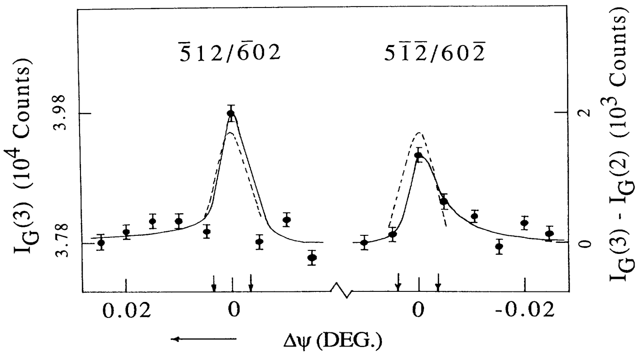

FIG. 2. Integrated three-beam intensities over ω vs $\Delta\psi$. The sense of crystal rotation and the positions at half maxima of the dashed I_K curves are indicated as arrows. The scale indicating the difference between the two-beam and three-beam intensities is also given.

with no anomalous dispersion, $I_D(a) = -I_D(b)$ and $I_K(a) = I_K(b) = [I'_G(a) + I'_G(b)]/2 = \bar{I}_K$ [20], where a and b refer to the $\bar{5}12/\bar{6}02$ and $5\bar{1}\bar{2}/60\bar{2}$ cases. Note that these two centrosymmetrically related cases have opposite signs in δ. Since the corresponding reflections involved in the two cases have almost the same strength, the extinction effects on the intensity measurements for both cases are similar. The scaling of $\bar{I}_K$ averaged over the two peak intensities should almost not be affected by the difference in extinction between the two cases. The constructed Gaussian peak tops for $I_K(\Delta\psi)$ with the FWHM of η and the peak value $\bar{I}_K$ are shown in Fig. 2 for comparison. From this figure and Eqs. (1) and (2) we immediately conclude that $\delta(a) < 0$ and $\delta(b) > 0$, since $I'_G(a) > I_K$, $I_D(a) > 0$ and $I'_G(b) < I_K$, $I_D(b) < 0$ at $\Delta\psi = 0$. The crystal polarity is thus determined.

Once I_K is known, the quantitative phase determination is straightforward [21] via

$$\tan\delta = -(I_+ + I_-)/(I_+ - I_-)\,, \tag{3}$$

where

$$I_\pm = I_D(\pm\eta/2) = (I'_G - I_K)_{\Delta\psi = \pm\eta/2} \tag{4}$$

and $I_K(\Delta\psi = \pm\eta/2) = \bar{I}_K/2$. The signs of $\sin\delta$ and $\cos\delta$ are the signs of the numerator and denominator of Eq. (3), respectively. Because of the deterioration of the crystal we were not able to perform ψ scans in smaller steps (because it was time consuming). Hence there are not many measured points near the peaks. Even so, the various curves fitted to the data points of Fig. 2 can still be used for quantitative phase analysis and error estimation. The triplet phases are determined from those curves (only a pair of curves are shown as a guide to the eye) via Eq. (3) as −135° ± 25° for $\bar{5}12/\bar{6}02$ and 134° ± 25° for $5\bar{1}\bar{2}/60\bar{2}$. The calculated phases from the known structure are −102° and 102°, respectively. The overall accuracy in δ is about 60°. Sharp intensity profiles of the resonance type, which are usually associated with 0° or 180° phases [3–6], were also observed. Similar curves were also obtained for $51\bar{2}/62\bar{2}$ on beam line X3A2 with $\lambda = 1.8$ Å. Figure 3 shows the profile of the $51\bar{2}/62\bar{2}$ (OUT) case. The sign of $\cos\delta$ is determined according to the sign rule given in Ref. [4] as $S(\cos\delta) = S_L$. $S_R = (-)(-) = +$, S_L and S_R being the signs defined from the profile asymmetry and the IN-OUT situation [4]. The phase value can be determined quantitatively, as described in Refs. [6,7], by quantifying the intensity asymmetry of the diffraction profile. The determined phase is 45° ± 20°, compared to the calculated 0°.

In conclusion, we have demonstrated that x-ray-reflection phases of large macromolecular crystals can be directly determined using three-beam interference. The analysis procedure presented, taking care of the extinctions, could lead to more reliable quantitative phase determination, if detailed ψ scans can be performed. Phase-determination experiments using three-beam diffraction for macromolecular crystals of unknown structure are planned for the next investigation.

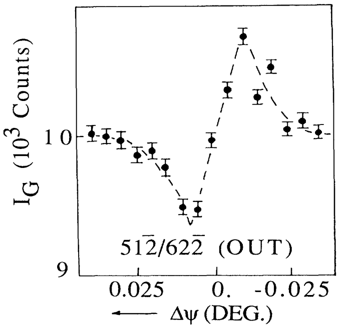

FIG. 3. Integrated intensities over ω vs $\Delta\psi$ of $51\bar{2}/62\bar{2}$ (OUT) for $\lambda = 1.8$ Å.

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