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A novel method of determining the number of macromolecules per asymmetric unit from accurate crystal-volume measurements

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Knowledge of the number of macromolecules per crystallographic asymmetric unit is frequently useful in the determination of crystal structures. A method has been developed to establish this number directly from measurements of the volume and macromolecular contents of a crystal. The volume of a crystal is determined by measuring the volume of solvent that it displaces in a fine capillary tube. The macromolecular mass contained in a crystal is measured by dissolving the crystal in a known amount of water or suitable buffer and then measuring the UV absorbance of the solution. The method has been tested successfully on three different crystals of known structures.

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1. Introduction

The number of macromolecules per crystallographic asymmetric unit (N_{au}) is often very valuable information in crystallographic structure determinations. It is among the elementary features of a crystal to be characterized after the crystal is grown (Drenth, 1994). N_{au} can assist in nearly every stage of structure determination. For instance, knowledge of N_{au} can assist in interpreting heavy-atom difference Patterson maps in MIR (multiple isomorphous replacement) and MAD (multiple-wavelength anomalous dispersion) phasing methods. Additionally, N_{au} can facilitate the identification of the correct solution of an MR (molecular-replacement) model search. Furthermore, N_{au} provides information about molecular packing used in non-crystallographic symmetry (NCS) averaging for *ab initio* phasing or structure refinement. N_{au} can also provide information about the oligomeric state and symmetry of some macromolecules.

The traditional approach for establishing N_{au} has been to measure the density of a crystal in a density gradient. This was first achieved by Low & Richards (1952) using a bromobenzene density gradient and subsequently by Westbrook using a Ficoll density gradient (Westbrook, 1976). In these methods, the density of a protein crystal must be measured accurately using a density gradient, which allows N_{au} to be derived from the partial specific volume of protein molecules. The density gradient needs to be calibrated by introducing a series of immiscible liquid drops of known density (Richards, 1992). Although it has played an important role in the development of crystallography, this approach has some undesirable features. Because solvent exchange between crystals and the Ficoll solution is inevitable in the process, the method often leads to erroneous results (Bode & Schirmer,

1985). Since these density-gradient methods are also inconvenient, they are not widely used in practice.

We have recently developed an accurate and convenient method to determine N_{au}. This method only concerns the macromolecular density in a crystal and not the combined density of macromolecules and solvent. To achieve this goal, we measure the total mass of macromolecules in the crystal from the UV absorbance of the dissolved crystal and measure the volume of the crystal using a volume-displacement method. We tested the method on three different types of crystals with success.

2. Methods

2.1. A direct method to determine the number of molecules per asymmetric unit, N_{au}

Because crystals can be regarded as a three-dimensional replica of their constituent unit cells, we can probe the contents of unit cells from features of crystals. The following equation relates the mass:volume ratio of the unit cell to that of the whole crystal:

$$M_{\rm protein_cell}/V_{\rm cell} = M_{\rm protein_crystal}/V_{\rm crystal}. \qquad (1)$$

Here, $M_{\rm protein_cell}$ is the mass of protein in a unit cell, $V_{\rm cell}$ is the volume of the unit cell, $M_{\rm protein_crystal}$ is the mass of protein in a crystal and $V_{\rm crystal}$ is the volume of the crystal. Both the $M_{\rm protein_cell}/V_{\rm cell}$ and the $M_{\rm protein_crystal}/V_{\rm crystal}$ ratios are the protein density in the crystal.

Since

$$M_{\rm protein_cell} = N_{\rm au} \times N_{\rm sg} \times {\rm MW}/N_{\rm avo}, \qquad (2)$$

then

$$N_{\rm au} = (M_{\rm protein_crystal} \times V_{\rm cell} \times N_{\rm avo})/(V_{\rm crystal} \times {\rm MW} \times N_{\rm sg}). \qquad (3)$$

In (2) and (3), N_{sg} is the number of asymmetric units in the unit cell, determined by the space group, MW is the molecular weight of the protein and N_{avo} is Avogadro's number.

The number of molecules per asymmetric unit, N_{au}, can be derived by determining the parameters in (3). N_{avo} is a known constant; the molecular weight, MW, can be derived from the protein (or nucleic acid) sequence or from an SDS denaturing gel; the unit-cell volume V_{cell} and the number of asymmetric units in the unit cell N_{sg} can be obtained from X-ray data. Thus, the number of molecules per asymmetric unit N_{au} can be determined from measurements of the volume and mass of the crystal. Following this logic, we have developed a volume-displacement method to measure the crystal volume $V_{crystal}$ and have used UV at 280 nm to measure $M_{\rm protein_crystal}$.

2.2. Measurements of crystal volumes by volume displacement in a fine capillary

Crystal volumes can be determined by measuring the volume of liquid that is displaced by a crystal in a fine capillary tube. The diameter of the capillary can be calibrated using a known volume of water. For instance, 10 µl of water is added to a capillary. Once the length occupied by a known volume of water in the capillary is measured under a microscope, the diameter of the capillary can be calculated. After calibration of the capillary diameter, the capillary is dried by filter-paper fibers.

To measure the volume of a crystal, a small amount of crystal mother liquor is added to the capillary. A crystal is then added into the capillary, initially without touching the mother liquor that is already in the capillary (Fig. 1*a*). Once all the mother liquor attached to the crystal is removed by filter-paper fibers or is air-dried, the volume of the mother liquor is calculated from measurement of the length of the capillary occupied by the mother liquor (Fig. 1*a*) and then the crystal is pushed into the existing mother liquor in the capillary (Fig. 1*b*). The total volume of the crystal and the mother liquor can again be calculated by measuring the length occupied by the liquid. The volume of mother liquor displaced by the crystal is the volume of the crystal. If the crystals are too fragile to be pushed into the mother liquor, the mother liquor can be pushed or dragged towards the crystal by carefully applying air pressure using an air bulb or a pipette from one end of the capillary.

We suggest that users of this method first estimate the dimensions of their crystals before choosing capillaries of appropriate sizes and selecting mother liquor of suitable volume. We recommend using a capillary whose diameter is

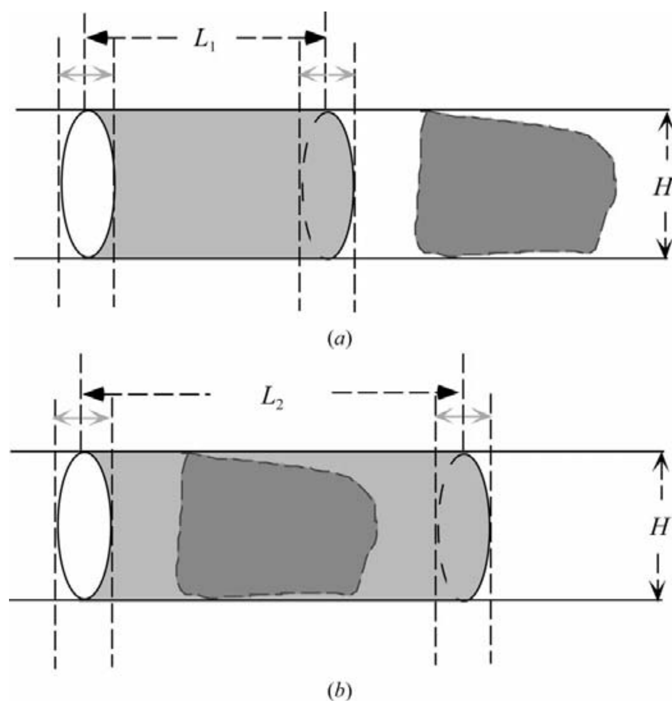

Figure 1
The volume-displacement method for measuring the volume of a crystal. (*a*) A small volume of crystal mother liquor is added into a fine capillary and a crystal is subsequently added without touching the liquid. The mother liquor attaching to the crystal is removed by fibers. (*b*) The crystal is pushed into the adjacent mother liquor. From the measured increase in length of the capillary occupied by the mother liquor and the known diameter of the capillary, the volume of the crystal can be calculated.

Table 1
Tests of the method on crystals of known structure (the typical sizes of the crystals tested here are 0.2–0.4 mm in each dimension).

Protein	Lysozyme	Klentro crystal form I	Klentro crystal form II
Space group	$P4_32_12$	$P2_12_12_1$	$P2_12_12_1$
Crystallization precipitant	0.8–1.0 *M* NaCl	15–17% PEG 8K	24% PEG 6K
Unit-cell parameters (Å)	$a = b = 79.1$, $c = 37.9$	$a = 85.3$, $b = 92.6$, $c = 93.7$	$a = 85.7$, $b = 93.9$, $c = 168.7$
N_{au} (theoretical)	1	1	2
N_{au} (measured)	0.97	0.98	1.92
No. of measurements	5	5	5
Standard deviation	0.06	0.08	0.13
95% confidence interval	(0.92, 1.02)	(0.90, 1.06)	(1.81, 2.03)

close to but slightly larger than the dimensions of the crystals. If crystals are small, one can choose capillaries with smaller diameters (*e.g.* 0.2 ml capillaries from Hampton Research, CA, USA) or measure the total volumes of several crystals in order to reduce errors in measurement. We also recommend that the volume of the mother liquor be 4–8 times greater than that of the crystal. In this study, measurements were successful using buffers containing up to 30% PEG 6K, suggesting that the method can handle the usual crystallization conditions, ranging from low to high (*e.g.* 30% PEG 6K) levels of viscosity. Outside the usual range, additional care and effort may be necessary to handle buffers with extreme concentrations of PEG or alcohol, in which case one may seek other crystal-stabilization buffers with lower viscosity. Finally, fibers or fine needles (*e.g.* from Hampton Research, CA, USA) can be used to efficiently dry small crystals in a capillary before the crystal volume is measured.

2.3. Measuring the mass of the protein in the crystal

After the volume of the crystal is measured, a small volume of water (or a suitable buffer that dissolves the crystal), *e.g.* 50 µl, is added into the capillary to dissolve the crystal and an additional amount of water (*e.g.* 50 µl) is used to wash the capillary. The absorbance of the total protein solution (in our example, 100 µl) at 280 nm is measured in a UV spectrometer. The amount of protein can then be calculated from its extinction coefficient. If the mass of the protein is small, we suggest using spectrophotometer cells with smaller volumes (*e.g.* a 10 or 50 µl cuvette from Starta, CA, USA). The Bradford method (Bradford, 1976), the Lowry method (Lowry *et al.*, 1951), the Edelhoch method (Gill & von Hippel, 1989) or amino-acid analysis are alternatives to the UV method for measuring the mass of the protein in the crystal. Once the volume and the mass of the crystal are measured, N_{au} can be uniquely calculated using (3).

3. Results

Using this method, we measured N_{au} in three different protein crystals: chicken lysozyme crystals (Blake *et al.*, 1965) and two different crystal forms of the large fragment of Tro DNA polymerase (Klentro; Li *et al.*, 2003). The typical sizes of the crystals tested are 0.2–0.4 mm in each dimension. After five measurements for each type of crystals, the 95% confidence interval of the N_{au} was 0.97 ± 0.05, 0.98 ± 0.08 and 1.92 ± 0.11 for each crystal type, respectively (Table 1). None of the estimated values was significantly different from its corresponding known N_{au}, which is 1, 1 and 2, respectively (all probabilities >0.20). In addition, all of the estimated values were significantly different from their closest alternative N_{au}: 2, 2 and 1 or 3, respectively (all probabilities <0.0001).

4. Discussion

Our method can be easily, accurately and quickly executed to determine the number of macromolecules within each asymmetric unit of a crystal. It can be applied not only to protein crystals, but also to crystals of nucleic acids and complexes of protein and nucleic acids after their extinction coefficients have been determined.

This method does not depend on a particular quality or shape of crystal. Crystals can have irregular shapes or be in the form of clusters because the volume measurement is not limited by these factors. This method can also be applied to small crystals that cannot be tested by the Ficoll density-gradient method. If crystals are too small, several crystals can have their total volume and total mass measured together to reduce errors in measurement. We have successfully tested the method on several crystals with dimensions of about 0.1 mm (errors less than 20%). The method requires no specialized equipment, since it can be performed using a light microscope and a UV spectrometer. Thus, we believe that this method has the potential for widespread use in crystallography laboratories.

Although we have successfully tested this method, its wide application can be facilitated if the procedure is automated by the development of special tools. These tools may include micrograduated cylinders for better crystal-volume measurement and computer-assisted analysis of volume measurement or UV-absorption measurement. If this is realised, the determination of N_{au} will be more automatic, speedy and accurate.

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