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# Application of a fine thread beam to the structure analysis of a hemihedrally twinned crystal of hydroxylamine oxidoreductase

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Accurate diffraction intensity data have been collected from a twinned $P6_3$ crystal of the 24-haem protein hydroxylamine oxidoreductase, from a nitrifying chemoautotrophic bacterium *Nitrosomonas europaea*, using synchrotron radiation at station BL6A of the Photon Factory. Estimation of the twinning fraction and deconvoluted intensity data, including native and heavy-atom derivative data, gave an improved Patterson function. Four diffraction data sets were collected from one crystal and an estimation of the twinning fraction to confirm the phenomena was undertaken. The successfully detwinned data sets were utilized in the structure analysis of the present enzyme. The mechanism of twinned-crystal formation is also discussed.

**Keywords: structures; hydroxylamine oxidoreductase; haems.**

## 1. Introduction

Increased research opportunities at synchrotron sources have permitted the use of the fine-tuned and focused beam for data collection from crystals that have unfavourable characteristics. Twinned crystals are fairly common in protein crystals. One of the causes of the problem of twinning is multiple stable packing of molecules in the crystal due to weak interactions among the neighbouring molecules (Fisher & Sweet, 1980; Gomis-Rüth *et al.*, 1995; Rees, 1980; Redinbo & Yeates, 1993; Reynolds *et al.*, 1985). To obtain diffraction intensity data from such crystals a thin beam from the synchrotron source is indispensable.

To keep the scattering waves from different domains separate, the domain size of a twinned crystal should be bigger than the size of the incident beam. The diffraction intensities from a twinned crystal can appear normal, but such a case is misleading (Yeates, 1997). The estimation of the twinning fraction with the assumption that the reflections are from a hemihedral twinned crystal, *i.e.* a weighted sum of two different reflections that are related within a twinning operation, allows the accurate measurement ('deconvolution') of the reflection intensity (Britton, 1972).

We have solved the X-ray crystal structure of hydroxylamine oxidoreductase (HAO) (Igarashi, Moriyama, Mikami & Tanaka, 1997) from an autotrophic bacterium, *Nitrosomonas europaea* (Hooper & Nason, 1965; Sayavedra-Soto *et al.*, 1994). The crystals used in the structure analysis were twinned. To isolate a single-crystal data set from the twinned-crystal data set, we developed a two-step procedure of refinement (Igarashi, Moriyama, Mikami & Tanaka, 1997) using Britton's method (Britton, 1972). The successful application of the two-step refinement on the HAO crystal gave an accurate twinning fraction. Based on these observations, we were able to obtain measured diffraction intensity data from a single-crystal portion of an HAO twinned crystal, *i.e.* with the fine-tuned and focused synchrotron radiation beam. We present these details here.

**Table 1**
Intensity data statistics before and after detwinning.

| Data† | Ra1 | Ra2 | Rb1 | Rb2 | SR1 | SR2 | SR3 | SR4 |
|---|---|---|---|---|---|---|---|---|
| $R_{sym}$ (%)‡ | 6.5 | 7.1 | 7.6 | 8.1 | 5.7 | 4.0 | 4.2 | 5.3 |
| Degree of twinning ($\alpha$) | 0.37 | 0.45 | 0.47 | 0.35 | 0.00 | 0.06 | 0.62 | 0.98 |

† Data name: Ra and Rb are data sets collected with a laboratory X-ray source with an R-AXIS IIc. Ra1 and Ra2 were collected from the same crystal, as are Rb1 and Rb2. SR1–SR4 were collected using the Photon Factory beam, from the same crystal, irradiated in different positions. ‡ $R_{sym} = 100S|\langle I\rangle - I_i|/S|I_i|$, over all symmetry equivalents on each frame between 40 and 3.0 Å resolution.

## 2. Crystallization and diffraction intensity data collection

HAO has a molecular mass of 200000 with 24 haems. It is an electron-transfer protein. The number of haems in a molecule is the largest among known haem proteins. As a result, the enzyme has a very dark red colour. HAO was crystallized by the hanging-drop vapour-diffusion method (Mikami *et al.*, 1991). The crystals grow with an ellipsoidal shape (the crystals are like rugby balls) with dimensions up to $0.4 \times 0.4 \times 0.8$ mm. The crystal space group is $P6_3$, with cell dimensions of $a = b = 96.2$ Å, $c = 265.7$ Å and $\gamma = 120^\circ$. X-ray intensity data up to 3.5 Å resolution were collected on an R-AXIS IIc and then merged using the program *PROCESS* (Higashi, 1990). The pseudo twofold axis is along [110], which runs along the diagonal between the *a* and *b* axes. However, the intensity distributions from different crystals are not consistent with one another although data sets can have a good internal *R* factor. These observations suggest that an HAO crystal is twinned, *i.e.* comprising two crystals together, looked at in exactly opposite directions. The merged reliability factors ($R_{merge}$) were 7.5%. The same observations were made for many crystals of the native protein and of the two heavy-atom derivatives prepared using $Hg(CH_3COO)_2$ or $K_2PtCl_4$.

The distribution of the two crystal lattices was examined by collecting intensity data at different positions of one crystal using a high-brilliance and finely focused beam (size 0.1 mm) with a Weissenberg camera at the Photon Factory, Tsukuba (Sakabe, 1991). Four sets of data were collected from one crystal by shifting the crystal by 0.2 mm per data set along the long direction of the crystal, which was finally found to be along the *c* axis. The frames were processed using the program *DENZO* (Otwinowski & Minor, 1993) and scaled together using the *CCP4* suite of programs (Collaborative Computational Project, Number 4, 1994).

## 3. Results and discussion

Twinning of HAO crystals is based on the following observations (Table 1). The isomorphous reliability factors ($R_{iso}$) between two data sets were high, typically at 35%. These variations were also observed between two data sets collected from one crystal. Also, the strong peaks of the self-rotation function occurred in a pseudo twofold axis orientation at 60 to 98% peak height with respect to the origin peak. The pseudo symmetry was confirmed by the Patterson function. Along [110] the twinned crystal lattices are

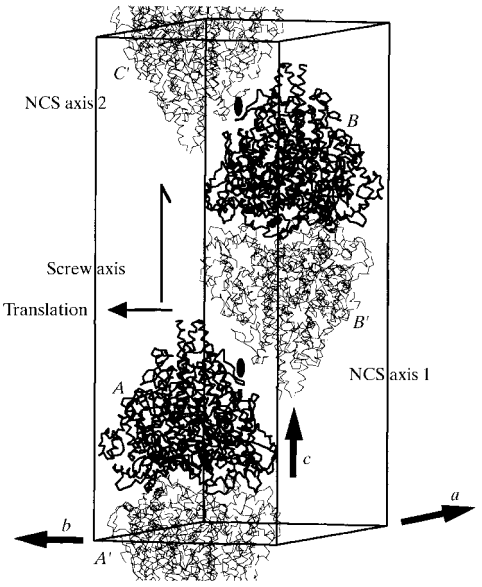


**Figure 1**
Molecular packing of the HAO crystal. In the crystal the molecules $A$ and $A'$, and $B$ and $B'$ are assembled. Molecules $AA'$ and $BB'$ are related by crystallographic symmetry. The 'ellipsoidal' symbol represents the twofold non-crystallographic symmetry (NCS) axis that runs perpendicular to the $c$ axis. The planes marked in black are interfaces between two molecular assemblies. If molecule $CC'$ moves as shown in the figure, the relationship between $BB'$ and $CC'$ cannot be distinguished from that of $BB'$ and $AA'$.

related to each other. The efficiency of the detwinning by means of the two-step least-squares method used by us (Igarashi, Moriyama, Mikami & Tanaka, 1997) was clearly seen in the difference Patterson function. On the Harker section of the difference Patterson function of the Hg derivative, calculated using the raw data, there were four prominent peaks from the heavy atoms. However, half of the peaks disappeared from the map calculated with the detwinned data and the additional symmetry observed in the self-rotation function disappeared. The twinning fractions were successively calculated with the data sets which were obtained from one crystal by using the tiny (0.1 mm) synchrotron radiation beam. For each data set, $R_{\rm sym}$ values, for equivalent reflections over a frame by frame of $100\Sigma|\langle I\rangle - Ij|/\Sigma|Ij|$, were between 40 and 5.7, and the scale factors, $G$, were between 0.82 and 1.36. $R_{\rm merge}$, for equivalent reflections over data set by data set of $100\Sigma|\langle I\rangle - Ij|/\Sigma|Ij|$, before and after the detwinning, were 45.5 and 3.8%, with the degree of twinning, $\alpha$, of 0.98 (= 0.02) at a tip of the crystal. At a middle point of the same crystal the corresponding values were 18.5 and 2.2% with $\alpha$ = 0.62. These results show that both lattices were truly mixed at the central part of the crystal. Hence, two crystal lattices can hemihedrally grow, *i.e.* in opposite directions to each other. True native and heavy-atom-derivative reflection intensities could be collected using this approach of checking the twinning fraction. Indeed, this was utilized successfully in the structure analysis of HAO (Igarashi, Moriyama, Fujiwara *et al.*, 1997). In data collection it is possible to obtain a single-crystal reflection data set from the tip of the crystal, so that the use of a thin beam with strong and parallel X-rays is indispensable in such a case.

The molecular basis of the twinning in the crystal is shown in Fig. 1. Two molecules are grouped together to form a bipyramid assembly. The assemblies are aligned to form a $2_1$ screw axis, which is parallel to the crystal $c$ axis. The neighbours along the screw axis are related by two possible non-crystallographic twofold axes. Because the oblique sides of a bipyramid along the longest axis have two different cross angles, it is possible to form two different directions for the screw axes. The nature of these screw axes are very similar in their intermolecular contacts due to the bipyramid assemblies having twofold symmetry. In fact, if a non-crystallographic symmetry axis rotates about the $a$ axis by 30° and then translates by 12.9 Å, then it can be on another non-crystallographic symmetry axis. This is the reason why this twin crystal type can occur. At the growing crystal surface, a newly orientating molecule is able to choose two different molecular interactions. The twinning is then the result of a small translation of the molecular assembly in the HAO crystal.

## 4. Concluding remarks

A finely focused beam of around 0.1 mm in size allows twin-free data to be collected from the tip of the crystal. Even in the middle of the crystal, twin fractions can be estimated. Synchrotron radiation was used to translate a crystal in 0.2 mm steps so as to estimate the twin fraction distribution along the length of the crystal. While it is shown that twin data could be 'deconvoluted', there is the potential to select portions of crystals that are twin-free with a finely focused synchrotron radiation beam, as demonstrated here.

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