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Structure Determination of Plastocyanin from a Specimen with a Hemihedral Twinning Fraction of One-Half

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Abstract

The structure determination of a macromolecule from a hemihedrally twinned crystal specimen with a twinning fraction of one-half is described. Twinning was detected by analysis of crystal-packing density and intensity statistics. The structure was solved using molecular replacement, and the positioned search model was used to overcome the twinning by a novel method of 'detwinning' the observed data. Estimates of the unobservable crystallographic intensities from each of the twin domains were obtained and used to refine the model. The structure of a new algal plastocyanin from *Chlamydomonas reinhardtii* was determined by this method to 1.6 Å resolution with a 'twinned' *R* factor of 15.6%. Additional data from a crystal specimen with a low twinning fraction were used to establish the accuracy of the structure solution from the perfectly twinned data, and to finalize the refinement to 1.5 Å resolution and a true *R* factor of 16.8%. Methods for detecting twinning and obtaining a molecular-replacement solution in the presence of twinning are discussed.

Introduction

Twinning is a crystal-growth anomaly in which a single specimen is composed of separate crystal domains the orientations of which differ in a special way. In merohedral twinning, the twin operation (the symmetry operation that relates the differently oriented domains of the specimen) is a member of the lattice symmetry but not a member of the point-group symmetry of the crystal. This is possible whenever the lattice is capable of supporting higher symmetry than the space group of the crystal. Merohedral twinning produces a diffraction pattern that is not visibly abnormal because the twinning operation exactly superimposes the reciprocal lattices of the separate domains.* Hemihedral twinning, the simplest and most common type of merohedral twinning, occurs when there are only two different orientations of the twin domains (Koch, 1992).

One of the consequences of hemihedral twinning is that each observed intensity is really the weighted sum of the intensities contributed by each of the two twin domains that make up the crystal specimen. Two reflections, $\mathbf{h}_1$ and $\mathbf{h}_2$, related by the twin operation but not by crystallographic symmetry, both contribute to two observed intensities:

$$I_{obs}(\mathbf{h}_1) = (1 - \alpha)I(\mathbf{h}_1) + \alpha I(\mathbf{h}_2) \quad (1)$$

$$I_{obs}(\mathbf{h}_2) = \alpha I(\mathbf{h}_1) + (1 - \alpha)I(\mathbf{h}_2) \quad (2)$$

where $0 \leq \alpha \leq \frac{1}{2}$.

The twinning fraction, α, represents the fractional volume of twin domain 2 relative to the entire specimen. A twinning fraction not equal to $\frac{1}{2}$ is referred to as 'partial' twinning, whereas a twinning fraction of exactly $\frac{1}{2}$ is termed 'perfect' twinning. In a perfectly twinned crystal specimen, the observed intensities are the average of the intensities from each of the twin domains. For the hemihedral case,

$$I_{obs}(\mathbf{h}_1) = I_{obs}(\mathbf{h}_2) = [I(\mathbf{h}_1) + I(\mathbf{h}_2)]/2. \quad (3)$$

This results in higher apparent symmetry, as the observed intensities of twin-related reflections become equal.

Determining a structure from a twinned crystal specimen is difficult, if not impossible, since direct measurement of the crystallographic intensities is not possible. The twinning problem can sometimes be avoided altogether by growing crystals in a different space group. Alternatively, if crystals with a low twinning fraction can be found, then (1) and (2) can be solved to give the crystallographic intensities, $I(\mathbf{h}_1)$ and $I(\mathbf{h}_2)$. However, as α approaches $\frac{1}{2}$,* (1) and (2) become degenerate [see (3)] and the distinct crystallographic intensities, $I(\mathbf{h}_1)$ and $I(\mathbf{h}_2)$, cannot be obtained from the two equivalent measured values, $I_{obs}(\mathbf{h}_1)$ and $I_{obs}(\mathbf{h}_2)$. Despite this obstacle, we show here that a macromolecular structure can, in fact, be determined by molecular replacement from a crystal

* Epitaxial twinning produces distinct reciprocal lattices because the twinning operation is not a member of the lattice symmetry.

* Solution of linear (1) and (2) amplifies errors in intensity measurement by a factor of approximately $1/(1 - 2\alpha)$.

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specimen with the highest possible twinning fraction, one-half. The structure of a novel plastocyanin from the green alga *Chlamydomonas reinhardtii*, determined from a perfectly twinned crystal specimen, is reported to 1.6 Å resolution. Additional data from a slightly twinned crystal specimen was used to assess the accuracy of this structure solution, and to finalize the refinement to 1.5 Å.

Detecting twinning

It is critical to identify twinning in a crystal specimen since unnoticed or misinterpreted twinning can prevent structure determination or lead to errors in the final structure solution. Merohedral twinning is seen only in lattice types where a cell or supercell of the lattice has higher symmetry than the true symmetry of the crystal point group. For biological macromolecules, partial twinning ($\alpha < \frac{1}{2}$) is seen with the 3, 32, 4, 6 and 23 point groups, while perfect twinning ($\alpha = \frac{1}{2}$) can give rise to the following higher apparent point-group symmetries: 32, 422, 6, 622 and 432 (Koch, 1992).

Twinned specimens can often be detected by microscopic examination, with a concave shape frequently indicating an intersection between separate crystals. In addition, differently orientated twin domains may exhibit different birefringence patterns when examined with crossed polarizers (Hartshorne & Stuart, 1969). One can detect partial twinning that is not revealed morphologically or optically by comparing the intensities of twin-related reflections. These intensities are not statistically independent in a partially twinned specimen, since they are related by (1) and (2). Statistical methods have been described to determine α for hemihedrally twinned specimens (Rees, 1982; Yeates, 1988; see also Britton, 1972; Murray-Rust, 1973; Fisher & Sweet, 1980).

In the special case of perfect twinning, twin-related intensities are not merely statistically dependent, but equal [see (3)]. The result is higher apparent symmetry which can easily be mistaken for true crystallographic symmetry. There are, however, several ways to identify perfect twinning. First, the apparent higher symmetry may indicate a number of molecules per unit cell that is impossibly high. For proteins, a value of 1.68 $Å^3$ Da^{-1} is an empirical lower limit for the Matthews coefficient (Matthews, 1968), the volume of the cell divided by the molecular weight of its contents. One can remove the uncertainty that arises from the wide range of allowable values for the Matthews coefficient by measuring the crystal density (reviewed in Westbrook, 1985). This provides an experimental measurement of the number of molecules in each unit cell. Second, the observed intensities can be tested for agreement with Wilson statistics (Wilson, 1949). Following Stanley (1972), the expected value of $\langle I^2(\mathbf{h})\rangle/\langle I(\mathbf{h})\rangle^2$ for acentric data in a thin resolution shell is 2.0 for cases without twinning and 1.5 for cases of perfect hemihedral twinning. Finally, when using multiple isomorphous replacement methods, heavy-atom cross vectors between positions related by the twinning operation will be absent.

For the structure reported here, precession photographs indicated that the space group was either $P6_2$ or $P6_4$, with cell dimensions $a = b = 61.8$, $c = 25.2$ Å, $\alpha = \beta = 90$, $\gamma = 120°$. A 1.6 Å data set was collected on an R-AXIS imaging plate and reduced in Laue group $6/m$ with an R_{merge} of 6.41%; no improvement was obtained by reducing the data in Laue symmetry $\bar{3}$. The Matthews coefficient was 1.32 $Å^3$ Da^{-1} for space group $P6_2$ or $P6_4$, and 2.64 $Å^3$ Da^{-1} for $P3_2$ (a subgroup of $P6_2$) or $P3_1$ (a subgroup of $P6_4$). The value $\langle I^2(\mathbf{h})\rangle/\langle I(\mathbf{h})\rangle^2$ for the structure reported here was 1.57 with an estimated uncertainty of 0.03 for data between 1.7 and 1.8 Å. This value remained constant over several resolution shells. From these results it was determined that the crystal specimen was perfectly twinned, and that the true space group was either $P3_1$ or $P3_2$. The space group was determined to be $P3_2$ by molecular replacement.

Molecular replacement with perfect twinning

It is possible to obtain a molecular-replacement solution from a perfectly twinned crystal specimen, provided the twinning is properly noted and accounted for. The symmetry of the rotation function is dictated by the higher symmetry of the observed diffraction data, and therefore the number of equivalent peaks in the rotation function is the order of the apparent point-group symmetry. For example, the apparent Laue symmetry of the twinned specimen reported here was $6/m$, whereas the true Laue symmetry was $\bar{3}$. According to the apparent symmetry, six equivalent peaks were obtained in a fast-rotation function (Crowther, 1972), differing only by an integral number of rotations of $\pi/3$ about the c axis (Fig. 1). The first, third and fifth rotation-function peaks comprise the equivalent molecular orientations in one $P3$ point group (twin domain 1), and the second, fourth and sixth rotation-function peaks comprise the molecular orientations in a second $P3$ point group (twin domain 2). Twin domain 1 is related to twin domain 2 by the twinning operation, a twofold rotation about the c axis. The first rotation-function solution was chosen and used in subsequent stages of molecular replacement. The molecular-replacement search model was the 1.85 Å crystal structure of *Enteromorpha prolifera* plastocyanin (Collyer, Guss, Sugimura, Yoshizaki & Freeman, 1990), which has 62% sequence identity to *C. reinhardtii* plastocyanin (Merchant *et al.*, 1990).

The translation function (Brünger, 1992) was run in the true crystallographic space group, rather than in the apparent higher symmetry space group produced by the perfect, hemihedral twinning. There should be no ambiguity as a result of twinning in such a translation function because a single rotation-function solution, and the orientations related by true crystallographic symmetry, represents only one twin domain. Since the apparent higher symmetry space group was either $P6_2$ or $P6_4$, the translation function was run in both $P3_2$ (a subgroup of $P6_2$) and $P3_1$ (a subgroup of $P6_4$). The data, which had been reduced after data collection to the asymmetric region of reciprocal space corresponding to Laue symmetry $6/m$, were expanded to the asymmetric region of reciprocal space corresponding to Laue symmetry $\bar{3}$ before running the translation function. The crystallographic space group, $P3_2$, gave the top translation-function solution ($T = 0.3919$, $\delta = 0.0843$), whereas the space group $P3_1$ gave no significant translation-function peaks ($T = 0.2236$, $\delta = 0.0028$).* $P3_2$ was indentified as the true space group in this way.

* T represents the top translation-function score. T is a weighted linear correlation between the observed and calculated normalized structure factors (Brünger, 1992). δ represents the difference between the top translation-function score and the next highest score.

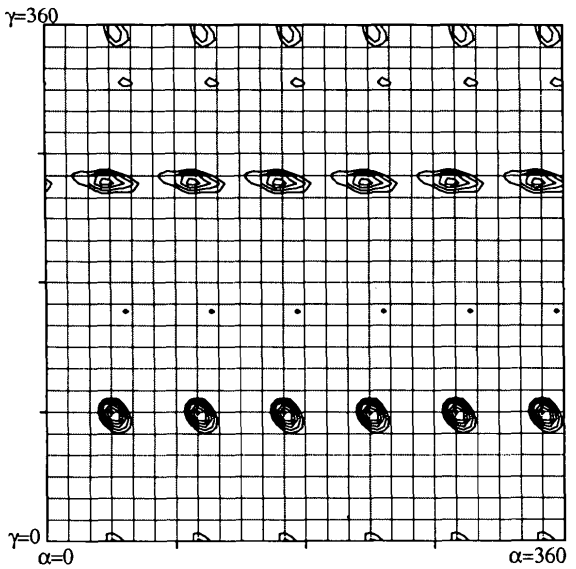

Fig. 1. The $\beta = 70°$ section of a cross-rotation function map (Crowther, 1972) using *Enteromorpha prolifera* plastocyanin as a search model. Diffraction data between 15 and 2.5 Å were included and the Patterson radius cut off was 15 Å. The map was contoured at 2, 2.5, 3, 3.5, 4 and 4.5σ. The peaks at $\gamma = 90°$ were the highest in the map. The periodicity of $\pi/3$ in α illustrates the apparent sixfold symmetry about the z axis for the observed data.

In principle, one could devise a new translation function that determines the optimum placement in the unit cell based on a comparison between the observed, twinned reflections, F_{obs}, and the calculated structure factors for the two twin domains, $[F^2_{calc}(\mathbf{h}_1) + F^2_{calc}(\mathbf{h}_2)]^{1/2}$, as a function of position in the unit cell. Such a new translation function, however, was not investigated.

Structural refinement with perfect twinning

There are two possible methods for refining a structure from a hemihedrally twinned specimen with $\alpha = \frac{1}{2}$. In one method, the quantities to be minimized would be the differences between the observed structure-factor amplitudes and values obtained by twinning the structure factors calculated from the current model, $[F^2_{calc}(\mathbf{h}_1) + F^2_{calc}(\mathbf{h}_2)]^{1/2}$. This method has been used successfully in small molecule crystallography (for example see Wei, 1969; van Koningsveld, 1983), but implementing it in existing macromolecular refinement programs did not appear to be straightforward. Furthermore, this procedure does not address the problem of calculating ordinary electron-density maps without knowledge of the true crystallographic intensities.

Instead we have developed a second method of refinement, in which we obtain estimates for the diffraction intensities from each of the separate twin domains. We refer to this process of obtaining diffraction intensities from twinned data as 'detwinning'. Recall that for a hemihedrally twinned specimen with $\alpha = \frac{1}{2}$, $I_{obs}(\mathbf{h}_1) = I_{obs}(\mathbf{h}_2) = I(\mathbf{h}_1) + I(\mathbf{h}_2)$* (3), where $I(\mathbf{h}_1)$ and $I(\mathbf{h}_2)$ represent the expected intensities contributed by each of the twin domains. $I_{obs}(\mathbf{h}_1)$ can be measured, but this provides only a single equation in terms of the two unknowns, $I(\mathbf{h}_1)$ and $I(\mathbf{h}_2)$. Additional equations can be obtained by approximating the untwinned diffraction intensities with values calculated from the positioned molecular-replacement model, $I_{calc}(\mathbf{h}_1)$ and $I_{calc}(\mathbf{h}_2)$:

$$I(\mathbf{h}_1) \simeq I_{calc}(\mathbf{h}_1) \qquad (4a)$$

$$I(\mathbf{h}_2) \simeq I_{calc}(\mathbf{h}_2), \qquad (4b)$$

for a model closely resembling the actual structure. Substitution into (3) gives

$$I(\mathbf{h}_1) = I_{obs}(\mathbf{h}_1) - I_{calc}(\mathbf{h}_2) \qquad (5a)$$

$$I(\mathbf{h}_2) = I_{obs}(\mathbf{h}_1) - I_{calc}(\mathbf{h}_1). \qquad (5b)$$

Thus, for each twin domain, two estimations of the untwinned intensities are given, by (4a) and (5a) for domain 1 and by (4b) and (5b) for domain 2. Least-squares minimization reduces these equation pairs to

* Ignoring an arbitrary factor of 2.

the arithmetic mean of the two estimates,

$$I_{\text{detwin}}(\mathbf{h}_1) = [I_{\text{obs}}(\mathbf{h}_1) + I_{\text{calc}}(\mathbf{h}_1) - I_{\text{calc}}(\mathbf{h}_2)]/2 \quad (6a)$$

$$I_{\text{detwin}}(\mathbf{h}_2) = [I_{\text{obs}}(\mathbf{h}_1) + I_{\text{calc}}(\mathbf{h}_2) - I_{\text{calc}}(\mathbf{h}_1)]/2. \quad (6b)$$

Using (6*a*) and (6*b*), the untwinned intensities from each of the twin domains can be estimated. These artificially 'detwinned' data are then used to refine the model and to generate electron-density maps.* The chosen molecular-replacement model is taken to be molecule 1, and thus is refined against $F_{\text{detwin}}(\mathbf{h}_1) = [I_{\text{detwin}}(\mathbf{h}_1)]^{1/2}$. As the model of molecule 1 improves through refinement and model building, $I_{\text{calc}}(\mathbf{h}_1)$ and $I_{\text{calc}}(\mathbf{h}_2)$ improve, which in turn improves the estimate of $I_{\text{detwin}}(\mathbf{h}_1)$ and facilitates further refinement. This manner of cyclical model improvement and detwinning is continued until structural refinement is complete.

Two different R factors can be calculated when refining in this way: (i) the R factor calculated using data against which the structure is being refined, which for molecule 1 would be

$$R_{\text{untw}} = \sum|F_{\text{calc}}(\mathbf{h}_1) - F_{\text{detwin}}(\mathbf{h}_1)|/\sum|F_{\text{detwin}}(\mathbf{h}_1)|, \quad (7)$$

and (ii) an R factor calculated against the original, twinned data, given by

$$R_{\text{tw}} = \sum|[F^2_{\text{calc}}(\mathbf{h}_1) + F^2_{\text{calc}}(\mathbf{h}_2)]^{1/2} - F_{\text{obs}}(\mathbf{h}_1)|/\sum|F_{\text{obs}}(\mathbf{h}_1)|. \quad (8)$$

R_{tw} in (8) relates the combined structure factors of both domains, $[F^2_{\text{calc}}(\mathbf{h}_1) + F^2_{\text{calc}}(\mathbf{h}_2)]^{1/2}$, to the original twinned data, $F_{\text{obs}}(\mathbf{h}_1)$, and involves no estimated parameters. R_{untw} in (7), on the other hand, compares the structure factors calculated from only a single orientation of the molecules, $F_{\text{calc}}(\mathbf{h}_1)$, to the artificially detwinned (non-experimental) data, $F_{\text{detwin}}(\mathbf{h}_1)$. Because R_{tw} is the less biased of the two R factors available, it was used to asses the progress of the structural refinement against the perfectly twinned data. R_{tw}, however, gives an underestimate of the residual error in the structure because the expected difference between the observed and calculated intensities is reduced by averaging over the two twin-related reflections. Specifically, the value for R_{tw} is lower than the expected value of a true crystallographic R factor by a factor of $2^{1/2}/2$. The true crystallographic R factor was used at a later stage, when data with a low twinning fraction became available.

Model building was accomplished with the program *FRODO* (Jones, 1978), and was accompanied by rounds of rigid-body refinement and positional refinement (based on structure factors) using *X-PLOR* (Brünger, 1992). Difference-density maps of the $2F_o - F_c$ and $F_o - F_c$ type were calculated using the detwinned data, $I_{\text{detwin}}(\mathbf{h}_1)$, from the current stage of refinement (Fig. 2). Atoms and residues were built into five-residue omit maps calculated using data detwinned with the corresponding five residues omitted from the detwinning step. For example, if an omit map was to be calculated for residues 1–5, then structure factors required to detwin the data were calculated from a model which lacked these residues. This removed any structural bias from the map that may have been present in the detwinned data. The first round of model building into omit maps was performed prior to any atomic refinement. After model building was complete, positional refinement and restrained and unrestrained B-factor refinement were performed, as well as two rounds of simulated annealing (Brünger, 1991). Following each stage of structural adjustment, the data were detwinned using the improved model, and refinement was continued with the newly detwinned data. In all, 12 cycles of detwinning were performed. 19 well resolved water molecules were added to the completed structure, as well as one large cation, tentatively identified as calcium. The final R_{tw}, calculated using (8), was 15.6% using all data from 8.0 to 1.58 Å (91% complete). The r.m.s. deviations from ideal bond lengths and angles were 0.013 Å and 2.73°, respectively.

Structural refinement with partially twinned data

After the structure determination using the perfectly twinned data was complete, a crystal specimen with a low-twinning fraction was obtained. The data collected from this specimen were reduced in space group $P3_2$ with an R_{merge} of 4.08%, compared to an R_{merge} of 46.6% for space group $P6_2$. This ruled out perfect twinning. The possibility of partial twinning was evaluated by calculating the twinning fraction (α) using two independent methods. First, the statistical approach of Yeates (1988) determined α to be 0.047. Second, a least-squares minimization of s and α in

$$sI_{\text{obs}}(\mathbf{h}_1) = (1 - \alpha)I_{\text{calc}}(\mathbf{h}_1) + \alpha I_{\text{calc}}(\mathbf{h}_2), \quad (9)$$

in which s is a scale factor, was used to obtain a value of 0.046 for α, with an estimated uncertainty of 0.01. The data were therefore detwinned using an $\alpha = 0.046$ and (1) and (2).

The final structure from the perfectly twinned solution was refined against these data. The true crystallographic R factor could now be used to assess the residual error in the model. After one cycle of rigid-body refinement in *X-PLOR* the true crystallographic R factor was 0.256. The quality of the electron-density maps was significantly improved by

* It can be shown that this second method of refinement is equivalent to the first method if the refinements are intensity-based least-squares minimizations (see *Appendix*).

the availability of the true crystallographic intensities. This effect is probably primarily a result of a twofold increase in the number of crystallographic intensities. The addition of one surface lysine side chain that was unresolved in the previous model and 35 water molecules was accompanied by one round of simulated annealing and ten rounds of positional and *B*-factor refinement. The *R* factor decreased to 0.187. Data to 1.5 Å were added, positional and *B*-factor refinement was continued, and electron-density maps were used to make minor structural adjustments. 26 additional water molecules were added, bringing the total number of solvent sites to 80. The final *R* factor is 16.8% using all data from 8.0 to 1.5 Å (92% complete). The deviations from ideality on bond lengths and bond angles are 0.020 Å and 1.88°, respectively.

The *R* factor calculated between the original perfectly twinned data and the data from the partially twinned specimen, perfectly twinned artificially by averaging the twin-related reflections (3), was 0.073. An R_{tw} calculated using this data, perfectly twinned artificially, and the structure factors calculated from the final model was 0.127. This value is lower than the true crystallographic *R* factor of 0.168 by a factor of approximately $2^{1/2}/2$, as expected. The r.m.s. deviation in the C^{α} backbones between the model solved from the perfectly twinned data and the final structural model was 0.125 Å.

It is of interest to consider how the 'detwinned' data used to refine the original structural model changed relative to the partially twinned data collected later. Before any detwinning step, the original perfectly twinned data (expanded to the asymmetric region of reciprocal space corresponding to Laue symmetry $\bar{3}$) had an *R* factor of 0.284 relative to the partially twinned data (detwinned with the low-twinning fraction of 0.047). After one round of detwinning the perfectly twinned data, the *R* factor relative to the partially twinned data dropped to 0.237. It continued to drop until it reached its final value of 0.194 after 12 cycles of detwinning. This suggests that the greatest improvement in the perfectly twinned data came in the first step of 'detwinning', and that subsequent steps improved the estimates of the crystallographic intensities to a lesser degree.

Concluding remarks

We have shown that it is possible to solve the structure of a macromolecule by molecular replacement using a hemihedrally twinned crystal specimen with a twinning fraction of one-half; this is the most difficult case. Cases of partial twinning ($\alpha \neq \frac{1}{2}$) can always be converted to 'perfect' twinning ($\alpha = \frac{1}{2}$) by averaging the twin-related intensities.

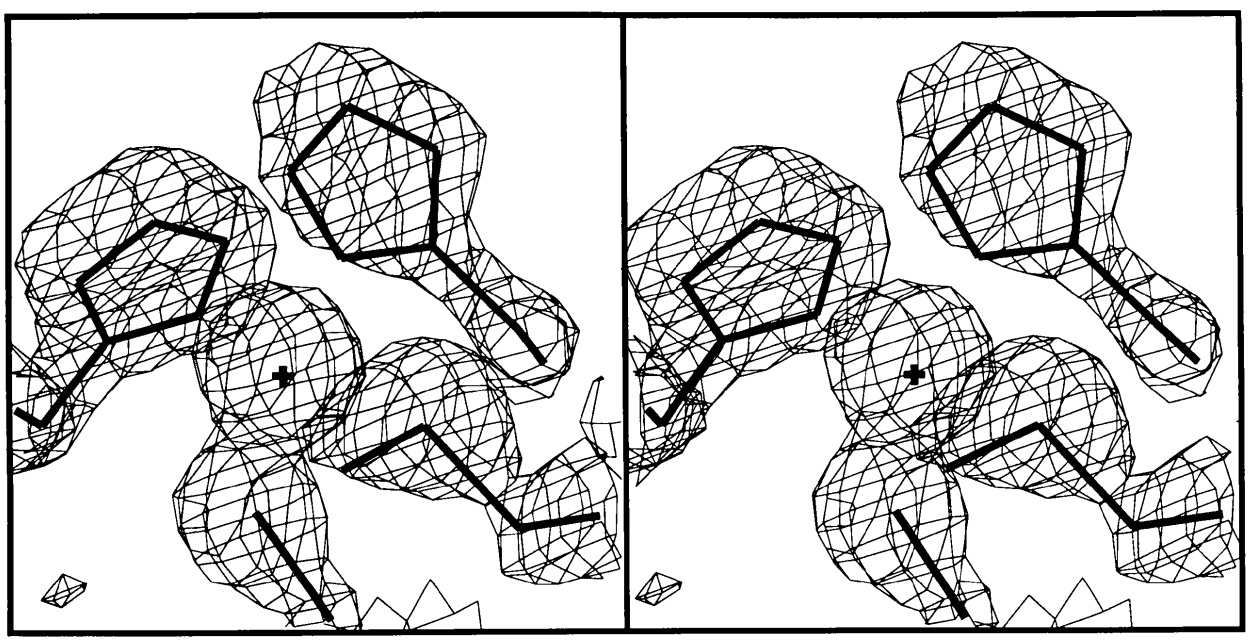

Fig. 2. Stereoview of an omit map at the copper site in plastocyanin. This map is based on data obtained from the perfectly twinned specimen. The Cu^{II} atom (represented by the cross) is liganded by two imidazole nitrogens from histidine side chains (from above), one methionine sulfur (in front) and one cysteine sulfur (from below). The map was contoured at 3σ and included data from 8 to 1.6 Å resolution. The remainder of the model was refined by simulated annealing prior to calculation of phases.

Plastocyanin from *Chlamydomonas reinhardtii* was crystallized in the apparent space group $P6_2$ (or $P6_4$), but density considerations and intensity statistics proved that the true space group was $P3_2$ (or $P3_1$). A molecular-replacement search model could be oriented and positioned without ambiguity in a $P3_2$ unit cell. This model was then used to obtain estimates of the true crystallographic intensities contributed by each of the twin domains, since these values cannot be directly measured. This novel procedure for 'detwinning' the observed data was followed by model building and atomic refinement, in cyclical fashion, in order to determine the structure of a new plastocyanin at 1.6 Å resolution. Additional data with a low twinning fraction extended the resolution to 1.5 Å and established that the structure solved from the perfectly twinned data was accurate. A complete report on the crystallization and structural details will be published elsewhere.*

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APPENDIX

Equivalence of two error functions for atomic refinement using perfectly twinned intensity data

In the text we introduce two possibilities for refining an atomic model when the diffraction data are perfectly twinned by hemihedry. If the minimizations are intensity-based, then the error function for the first method would be

$$E_1 = \sum_{\mathbf{h}_1} \{I_{\rm obs}(\mathbf{h}_1) - [I_{\rm calc}(\mathbf{h}_1) + I_{\rm calc}(\mathbf{h}_2)]\}^2, \qquad (a)$$

where $\mathbf{h}_2$ is related to $\mathbf{h}_1$ by twinning. $I_{\rm calc}$, and therefore, E_1, is a function of atomic positions. In the second approach (see text), an intensity-based error function would be

$$E_2 = \sum_{\mathbf{h}_1} [I_{\rm detwin}(\mathbf{h}_1) - I_{\rm calc}(\mathbf{h}_1)]^2.$$

Substituting the expression for $I_{\rm detwin}(\mathbf{h}_1)$ from (6*a*) gives

$$E_2 = \sum_{\mathbf{h}_1} \langle (1/2)\{I_{\rm obs}(\mathbf{h}_1) - [I_{\rm calc}(\mathbf{h}_1) + I_{\rm calc}(\mathbf{h}_2)]\}\rangle^2, \qquad (b)$$

which shows that E_2 is clearly proportional to E_1. Therefore, intensity-based minimizations by the two methods are equivalent.

* Atomic coordinates and structure factors have been deposited with the Protein Data Bank, Brookhaven National Laboratory (Reference: 2PLT, R2PLTSF). Free copies may be obtained through The Technical Editor, International Union of Crystallography, 5 Abbey Square, Chester CH1 2HU, England (Supplementary Publication No. SUP 37085). A list of deposited data is given at the end of this issue.

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