Structure Solution of Azurin II from *Alcaligenes xylosoxidans* using the Laue Method: Possibility of Studying *In Situ* Redox Changes using X-rays

M. J. Debenham,[a,b] **Q. Hao,**[a]* **S. S. Hasnain,**[b]* **F. E. Dodd,**[a,b] **Z. H. L. Abraham**[c] **and R. R. Eady**[c]

[a]*School of Applied Sciences, De Montfort University, Leicester LE1 9BH, UK,* [b]*Molecular Biophysics Group, Daresbury Laboratory, Warrington WA4 4AD, UK, and* [c]*Nitrogen Fixation Laboratory at University of Sussex, Brighton BN1 9JQ, UK*

We have recently demonstrated that X-rays can be used for changing the redox states of the metal centre in metalloproteins [Murphy *et al.* (1995). *J. Synchrotron Rad.* **2**, 64–69]. The possibility of using the Laue method for studying the structural changes associated with such X-ray-induced reactions is explored by applying the method to the structure determination of a new azurin (hereafter referred to as azurin II) from the denitrifying bacterium *Alcaligenes xylosoxidans*. Laue X-ray diffraction data of azurin II were collected at station 9.7 of the SRS Daresbury. Three diffraction patterns were recorded on film packs at three different crystal orientations. The data were processed using the Daresbury Laue Software Suite to give 2224 independent single reflections (R_{merge} = 0.136) in the wavelength range 0.36–1.40 Å. The data completeness was 44% at 2.55 Å resolution. Phase determination for the data was undertaken using the molecular-replacement method; the top peak was chosen in both the rotation function and the subsequent translation function. This solution agreed well with the molecular-replacement solution achieved independently using monochromatic data. The electron-density map showed reasonably good agreement with the model and the copper site was readily recognizable as it had the highest density. To see if the electron-density map could be improved, the doublets in the diffraction data were then deconvoluted. This added 26% data in the region $\infty - 2d_{min}$ resulting in an improvement in the data completeness to 50% and thus in improved continuity of the electron-density map. The quality of these maps is discussed from the point of view of the suitability of this approach for studying redox-induced structural changes.

Keywords: azurin; Laue method; denitrification; electron transfer; redox reaction; time-resolved studies; data completeness.

1. Introduction

Nature utilizes redox states of metal centres in biological molecules to perform a variety of life-sustaining functions. The movement of electrons (or the change in redox state) is accompanied by structural changes. In some cases, the changes are subtle and restricted at or around the metal site (Guss & Freeman, 1983; Guss, Harrowell, Murata, Norris & Freeman, 1986; Norris, Anderson & Baker, 1983; Shepard, Anderson, Lewandowski, Norris & Baker, 1990; Groeneveld *et al.*, 1986; Strange, Reinhammar, Murphy & Hasnain, 1990), but in other cases major structural changes take place (Fülöp, Moir, Ferguson & Hajdu, 1995; Berger & Wharton, 1980). A change in redox state can be induced by a variety of chemical and electrochemical means. Recently, we have demonstrated that the copper centre in a small blue copper protein in solution can be reduced by intense monochromatic X-rays available from synchrotron radiation sources (Murphy *et al.*, 1995).

The Laue method has gained renewed interest recently with the advent of synchrotron radiation and advanced computational techniques (Moffat, Szebenyi & Bilderback, 1984; Hajdu *et al.*, 1987; Helliwell *et al.*, 1989). It has been shown that protein structures can be determined using Laue data and the molecular-replacement method (Howell, Almo, Parsons, Hajdu & Petsko, 1992). The advantage and great potential of the Laue method of data collection is the speed with which a large proportion of the data set can be measured. This has led to the use of the Laue method for time-resolved studies of crystal structures (Hajdu, Acharya, Stuart, Barford & Johnson, 1988; Schlichting *et al.*, 1990; Allinson *et al.*, 1992; Szebenyi *et al.*, 1992; Singer, Smalås, Carty, Mangel & Sweet, 1993). In particular, Singer *et al.* (1993) have revealed the hydrolytic water molecule in trypsin by time-resolved Laue crystallography.

Usually, 80–90% of the spots in a Laue diffraction pattern correspond to single reflections, the remaining 10–20% of the spots are doubles or higher multiples (Cruickshank,

Helliwell & Moffat, 1987). For multiples, the measurement of the spot intensity does not directly give the intensities of each individual reflection. Moreover, the reflections that cannot be straightforwardly measured as singles are not randomly distributed in reciprocal space; a high proportion of them are low-order reflections. The absence of these reflections can cause poor connectivity in electron-density maps (Duke *et al.*, 1992). There has been increasing interest in methods to deconvolute reflection intensities from spots that are multiples (Helliwell *et al.*, 1989; Campbell & Hao, 1993; Hao, Campbell, Harding & Helliwell, 1993; Hao, Harding & Campbell, 1995*a*; Ren & Moffat, 1994; Campbell *et al.*, 1994). Here, we use the real-space density modification method *DECONV* (Hao, Harding & Campbell, 1995*a*), which is incorporated in the Daresbury Laue Suite (Helliwell *et al.*, 1989).

In this paper we present Laue crystallographic structure determination of azurin II, a new azurin recently identified in a denitrification bacterium *Alcaligenes xylosoxidans*. In this bacterium we have recently identified two azurins, azurin I being that previously identified (Suzuki & Iwasaki, 1962), and azurin II being a new azurin (Dodd, Debenham *et al.*, 1995). Both of these azurins have been found to be able to donate electrons to the copper-containing nitrite reductase of this organism, a key enzyme in the denitrification pathway. Azurin II forms blue elongated rectangular prisms and crystallizes in the tetragonal space group $P4_122$ with unit-cell parameters $a = b = 52.65$, $c = 100.63$ Å. There is one molecule (129 residues) in the asymmetric unit with molecular weight 13 759 Da. The motivation of this study stems from the fact that the Laue method has the potential for time-resolved studies and, in the case of azurin, X-rays from the synchrotron source can themselves be used to change the redox state of the protein. Here, the Laue method is used to determine the structure of azurin II in order to assess the feasibility of using this approach for defining the structural changes that may occur upon a change in the redox state. As in real time-resolved kinetic experiments, the number of exposures is likely to be limited; we have used only three orientations to obtain data to test its adequacy for structure determination. The molecular-replacement solution obtained with the Laue data agreed well with that achieved independently using monochromatic data (Dodd, Hasnain, Abraham, Eady & Smith, 1995). The structure of azurin II has also been determined by Inoue *et al.* (1994), who also provide evidence for the existence of two azurins in this organism.

2. Data collection and processing

Laue diffraction patterns for azurin II were recorded on station 9.7 located on the 5 T wiggler of the Synchrotron Radiation Source (SRS) at Daresbury Laboratory. The incident beam was attenuated by 0.2 mm Al foil in order to reduce possible radiation damage. Data were collected on film packs, each composed of six films, on a crystal of dimensions $0.5 \times 0.07 \times 0.07$ mm at three orientations

(14, 20, 30°). These orientations were chosen to maximize the coverage of reciprocal space with the minimum number of orientations. This was done to mimic the situation of a time-resolved redox reaction where significantly longer X-ray exposure would result in converting the redox centre to the final state. The exposure time was 5 s with the SRS running at *ca* 200 mA. The crystal-to-film distance was 132 mm and the diameter of the exposed area on the film was 120 mm. Films were scanned on a microdensitometer with a 50 µm raster size. An example of a Laue diffraction photograph is shown in Fig. 1. The data were processed using the Daresbury Laue Suite (Helliwell *et al.*, 1989) with X-window-based *LAUEGEN* (Campbell, 1995). Soft limits λ_{min} and d_{min} (data resolution) were estimated by the intensity histogram method (Hao, Harding & Campbell, 1995*b*) to be 0.36 and 2.55 Å, respectively; λ_{max} was estimated to be 2.0 Å by comparison of observed and predicted patterns. Crystal orientations were found and refined and spot intensities integrated using *INTLAUE* (Helliwell *et al.*, 1989). Spots closer than 0.4 mm were classed as spatial overlaps. The r.m.s. deviations between observed and predicted spot positions were *ca* 0.03 mm for all the films. The intensities from the different films within each film pack were then scaled using *AFSCALE* (Helliwell *et al.*, 1989) with merging R between 8–10%. *LAUENORM* (Helliwell *et al.*, 1989) was used for wavelength normalization. Intensity measurements for 6205 singles with $I > 1.5\sigma(I)$ in the wavelength range 0.36–1.40 Å yielded 2224 unique reflections with R_{merge} (on I) = 0.136. The completeness of the data for ∞–d_{min}, ∞–$2d_{min}$ and $2d_{min}$–d_{min} was 44, 10 and 50%, respectively.

Reflection intensities for the components of double Laue spots were then deconvoluted using *DECONV* (Hao, Harding & Campbell, 1995*a*); 624 doubles with $I > 2.0\sigma(I)$ yielded 345 unique reflections with R_{merge} (on I) = 0.226. The deconvoluted higher multiples ($n \geq 3$) were not included due to higher R_{merge}. The combination of singles and doubles gave a total of 2480 independent reflections. The completeness of the data was improved to 50, 36 and 52% for ∞–d_{min}, ∞–$2d_{min}$ and $2d_{min}$–d_{min}, respectively.

3. Structure determination and refinement

The molecular-replacement program *AMoRe* (Navaza, 1994) was used to determine the structure of azurin II with the Laue data. The coordinates of the refined model of azurin of *Alcaligenes denitrificans* (Baker, 1988) obtained from the Brookhaven Data Bank (Bernstein *et al.*, 1977; accession number 2AZA) were used as the search model. There are 14 amino-acid differences between the search model and azurin II (129 amino acids in total) (Dodd, Debenham *et al.*, 1995). The solution was first attempted using the singles data alone. Rotational searches were carried out by a systematic reorientation of the Patterson function of the search model and a search for its maximum overlap with the Patterson function computed from the azurin II Laue data. The rotation function was scaled
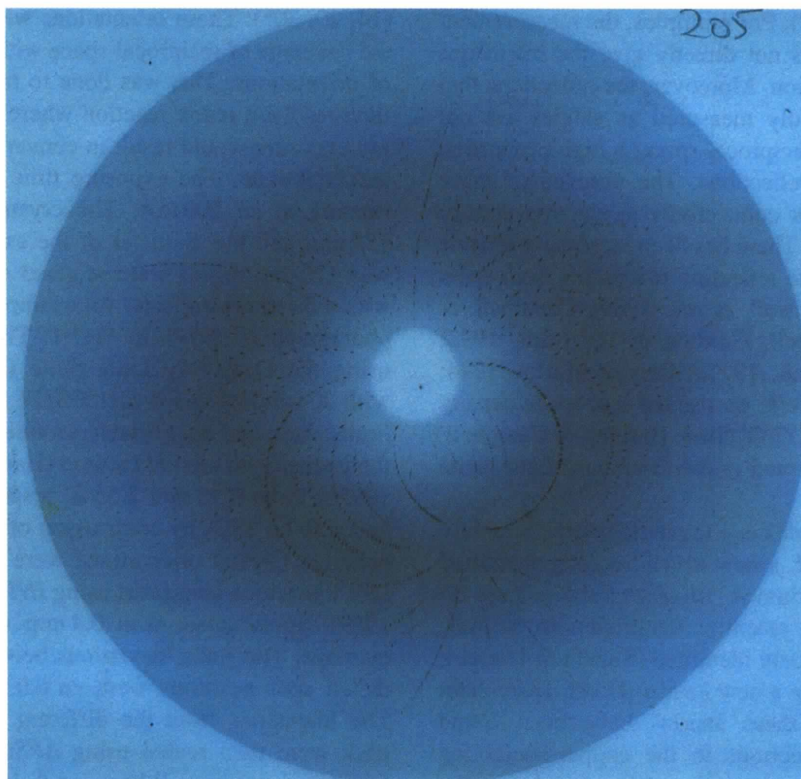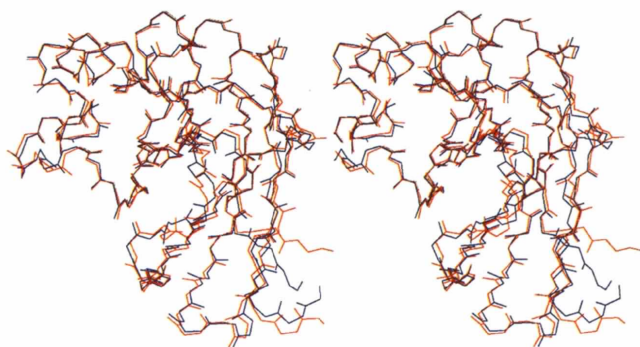
Figure 1
An example of a Laue diffraction photograph of azurin II recorded at station 9.7 of the SRS Daresbury.
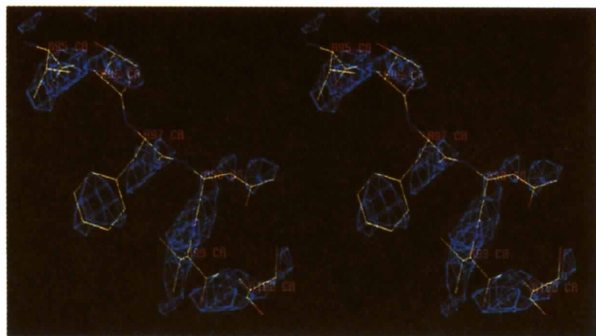
(a)
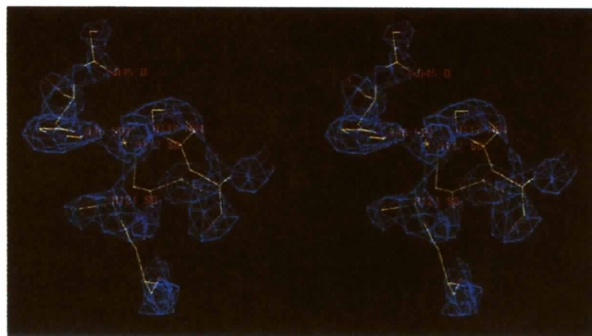
(c)
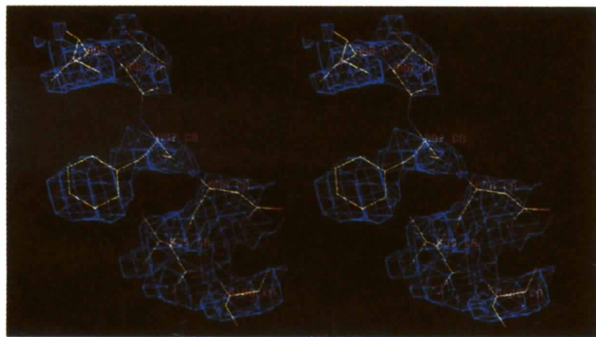
(b)
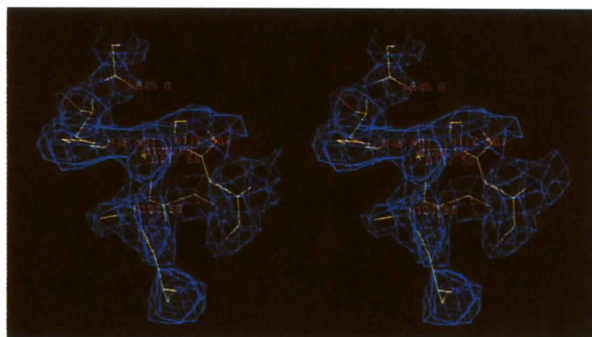
(d)

Figure 2
Electron-density maps of azurin II for two segments of the molecules, obtained with singles-only data and singles-plus-doubles data. (a) and (b) compare the two maps for the peptide region 95–100, while (c) and (d) compare the Cu site for singles-only data and singles-plus-doubles data, respectively.
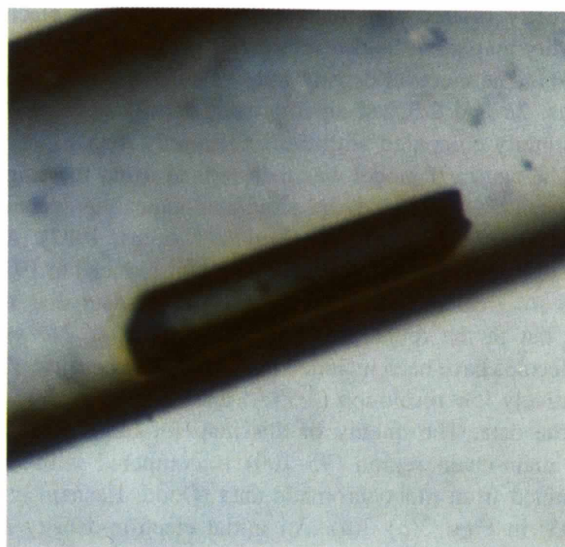
(a)

(c)

(b)

(d)
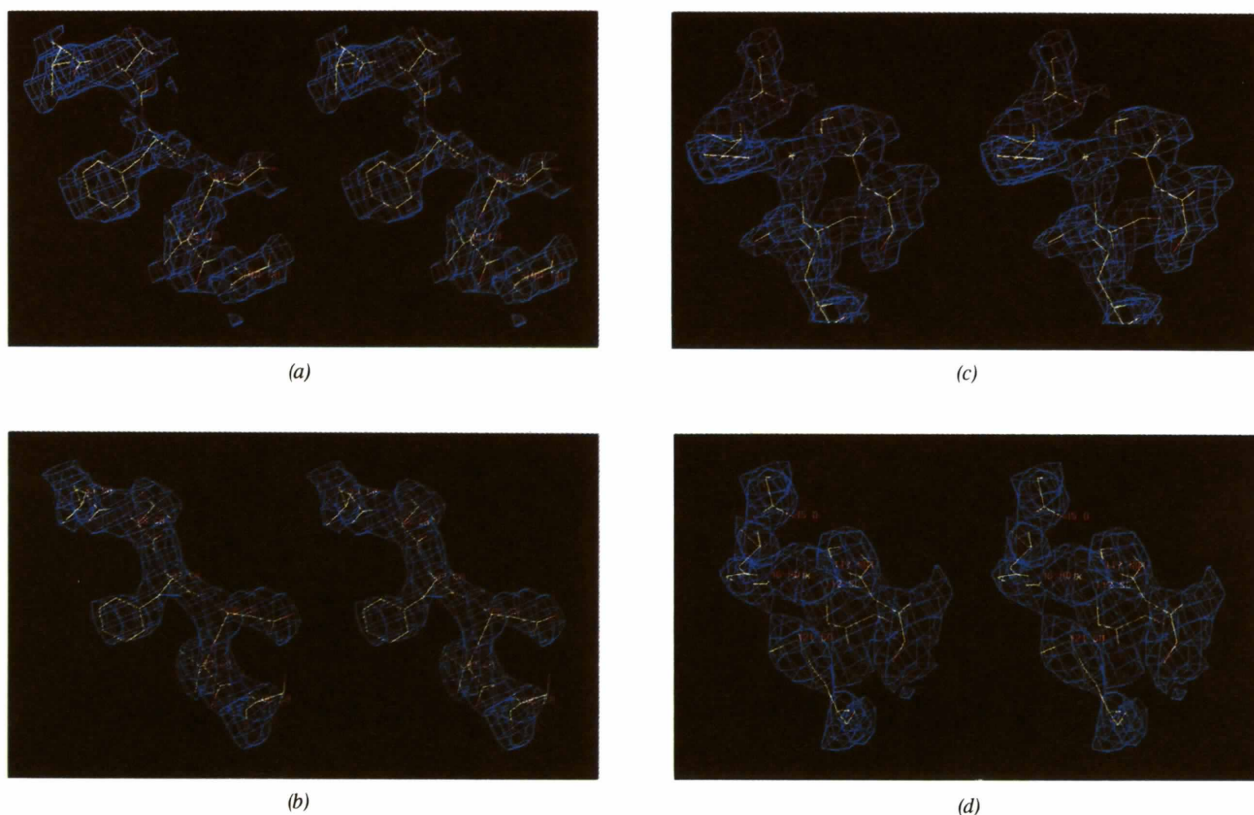
Figure 3
Electron-density maps, (a) and (b), of the peptide region (95–100) obtained after one cycle of refinement with the Laue (singles-plus-doubles) data and monochromatic data, respectively. (c) and (d) are equivalent maps for the Cu site. All Laue maps (including those in Fig. 2) are contoured at the same level (10% of the Cu peak), while the monochromatic map is contoured at 20% of the Cu peak.

Figure 4
A stereoview of the superposition of the backbone of azurin II determined using the Laue (red) and monochromatic (dark blue) data.

Figure 5
A photograph of the azurin II crystal from which Laue photographs were made. Note the clear loss of intense blue colour from the exposed part of the crystal.

from 1–10. The highest peak with a scaled value of 10.0 was found at $\alpha = 51.15$, $\beta = 67.17$, $\gamma = 210.83°$, which was chosen as the correct orientation. The next highest peak had a value of 9.6. Since the preliminary characterization (Dodd, Debenham *et al.*, 1995) of the space group had shown it to be either $P4_122$ or its enantiomorph $P4_322$, calculations for both had to be undertaken. The rotation-function output for both of these is identical, as expected. Calculation of the translation function allowed the

correct space group to be determined. When the translation function maps were produced it could be clearly seen that the space group $P4_122$ was correct for this crystal.

This is the same as that obtained independently using the monochromatic data (Dodd, Hasnain *et al.*, 1995). The Laue map showed a single peak (correlation coefficient = 64.7%, R = 36.7%) that was significantly higher than all the others (the next highest peak had correlation coefficient = 49.3%, R = 42.2%). For the other space group $P4_322$, there were many peaks of similar heights (correlation coefficient $\simeq$ 30%, $R \simeq$ 48%) at the top of the peak list. *AMoRe* (Navaza, 1994) then performed the rigid-body fitting and conversion of the solutions to the final model. The R factor of the solution after rigid-body refinement was 27.3%. The search model was rotated and translated into the azurin II unit cell. *Mapcalc* (Collaborative Computational Project, Number 4, 1994) was used to obtain the $(2F_o - F_c)$ maps. The modelling program O (Jones, Zou, Cowan & Kjeldgaard, 1991) was then used to change the 14 amino-acid differences between the search model to those of azurin II. The quality of the electron-density maps is shown for a typical segment of the main chain (Fig. 2a) and the copper site (Fig. 2c). The density coverage is reasonable; however, there are some discontinuities in the density coverage due to the lack of low-resolution data and a low data completeness of 44%.

To see if the density map could improve by including deconvoluted data from doubles, the molecular-replacement procedure was also carried out using the combined singles and doubles data. In this case, 26% data were added in the region ∞–$2d_{min}$ and 6% overall. The same solution to that for the singles-only data was achieved in both rotation and translation function searches. The R factor of the solution after rigid-body refinement was 30.2%. The correctly rotated and translated model and the electron-density map were loaded into the O program (Jones *et al.*, 1991). The electron-density map calculated with this data (Figs. 2b and 2d) had an improved density coverage and continuity compared with the singles-only map.

The azurin II model was then refined using the singles-plus-doubles data and the simulated-annealing technique with *XPLOR* (Brünger, Kuriyan & Karplus, 1987). After one cycle of refinement, the R factor was reduced to 19.1%. The R_{free} calculated from the 5% of the data that were left out in the refinement process was 27.0%. No water molecules have been included in the structure because of the relatively low resolution (2.55 Å) and completeness (50%) of the data. The quality of this map for the Cu site and the main-chain region (95–100) is compared with those obtained from monochromatic data (Dodd, Hasnain *et al.*, 1995) in Figs. 3(a)–3(d). An initial electron-density map obtained from 2.5 Å monochromatic data after one cycle of refinement is compared with the 2.55 Å Laue singles-plus-doubles map in order to directly assess the quality of the two maps. It is clear that despite the improvement due to the addition of doubles data, the quality of the 2.5 Å monochromatic map is significantly superior.

In a concurrent but independent study, the structure of azurin II has also been solved with monochromatic data extending to 1.9 Å (Dodd, Hasnain *et al.*, 1995). The final

R factor for 12162 monochromatic observations between 8.5 and 1.9 Å was 18.8% with R_{free} 20.7%. Despite the difference in resolution, an analysis of the r.m.s. deviations in all backbone atoms between the monochromatic structure and the structure determined with Laue data shows that the two structures agree well to within 0.7 Å. A stereoview of the two structures is shown in Fig. 4.

4. X-ray-induced change of redox state

Fig. 5 shows a photograph of the azurin II crystal that was used to collect the Laue data. The intense blue colour evident in the unexposed half of the crystal is largely lost in the lower half of the crystal showing the conversion of Cu^{2+} to Cu^+. The azurin II crystal diffracted to similar resolution for the three consecutive Laue pictures. The loss of colour without a significant change in the diffraction limit suggests that the photoreduction takes place before the loss of crystalline order and thus intense X-rays can be used for probing the redox state *in situ* in crystalline systems.

5. Discussion

We have shown that Laue diffraction data are capable of being used for protein structure determinations using the molecular-replacement technique even with low completeness of data. However, the successful solution of azurin II by the molecular-replacement method is partly due to a high homology (89%) and the well refined nature of the search model. More complete data would be needed in general cases where homologies are less favourable.

The main advantage of the Laue method is that the data-collection time is short. The possibility that the redox state of a metal centre in metalloproteins can be altered by the use of X-rays opens the tantalizing possibility of capturing structural changes which accompany the redox change in real time by using the Laue method. However, as the structural changes in azurins or similar proteins, which may accompany change in the redox state, are expected to be small (< 0.4 Å), particular attention is required in designing the Laue experiment if one is to be successful in defining these structural changes with sufficient precision and confidence. For example, improvements can be obtained by increasing the completeness of data by using a larger detector or increased number of crystal settings. The overall quality of data can be improved by using better detectors and better deconvolution of higher multiples (which give R_{merge} on these reflections under 15%). Some of these improvements have already been discussed by other workers (Cassetta *et al.*, 1993; Ren & Moffat, 1994). Work is currently in progress incorporating the above-mentioned improvements. The use of X-rays to induce the redox reactions is novel and should be explored with other synchrotron-radiation-based techniques.

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