Crystallization and preliminary diffraction data analysis of both single and pseudo-merohedrally twinned crystals of rubredoxin oxygen oxidoreductase from *Desulfovibrio gigas*

Crystals of rubredoxin oxygen oxidoreductase have been obtained and characterized. They belong to space group *P*2₁2₁2₁, with unit-cell dimensions *a* = 88.24 (15), *b* = 101.25 (7), *c* = 90.80 (3) Å. The homodimer (86 kDa) in the asymmetric unit is related by a non-crystallographic twofold rotation axis parallel to the *ab* `diagonal' direction, as shown by the self-rotation maximum in the section with *y* = 180°. This pseudo-crystallographic symmetry element was also found to be the twinning axis of pseudo-merohedrally twinned crystals, leading to apparent pseudo-tetragonal *P*4₂₂ crystal symmetry.

1. Introduction

*Desulfovibrio gigas* is a sulfate-reducing bacterium which is able to utilize polyglucose for the formation of ATP associated with the reduction of dioxygen to water (Santos et al., 1995). Rubredoxin oxygen oxidoreductase (ROO), an 86 kDa homodimer (Chen et al., 1993a), is the terminal element of a soluble electron-transfer chain in *D. gigas*, where electrons flow from NADH to oxygen through NADH-rubredoxin oxidoreductase (Chen et al., 1993b) and rubredoxin (LeGall & Dragoni, 1996). ROO contains flavin as a prosthetic group (Timkovich et al., 1994). Spectroscopic studies (Gomes et al., 1997) showed that the flavins accept electrons from rubredoxin. These data have been further supported by the finding that rubredoxin and ROO are located in the same polycistronic unit of the *D. gigas* genome. However, since the direct reduction of oxygen to water by flavins is unprecedented, the presence of another redox centre, as yet undetected, cannot be ruled out.

A twinned crystal consists of a specimen containing two or more single crystals of the same species, but in different orientations (Koch, 1995). In contrast to epitaxic twins, in which the twin components have unrelated orientations and therefore produce rotational images showing the superposition of their lattices, merohedral twins are formed by domains whose lattices overlap perfectly in three dimensions. Their diffraction patterns seem to be that of a single crystal, but the observed diffraction data do not represent the true crystallographic intensities: each spot is an overlap of different reflections from the different domains (which coincide because the twinning operator belongs to the underlying apparent crystal point group). Cases where the lattices of the different components overlap approximately (but not exactly) in three dimensions owing to fortuitous unit-cell geometry are referred as pseudo-merohedral.

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2. Materials and methods

2.1. Protein crystallization

Purified native ROO was obtained as described by Chen et al. (1993a). Screening for crystallization conditions indicated that ROO crystallizes by vapor diffusion at room temperature in the pH range 5–9, using polyethylene glycols (PEGs) in the molecular-weight range 1–6 K as precipitating agents. Crystals appear within 1 d, mostly in very large numbers, together with a gelatinous precipitate from which they are difficult to separate. The gelatinous precipitate, however, can be avoided by lowering the temperature and/or by addition of a detergent additive, SB12 (N-dodecyl-N,N-dimethyl-3-ammonio-1-propanesulfonate, Sigma Chemical Co.). In a typical crystallization trial, a sitting drop consisting of
Crystallographic statistics of rubredoxin oxygen oxidoreductase.

<table>
<thead>
<tr>
<th>Crystal growing conditions</th>
<th>Crystal A</th>
<th>Crystal B</th>
<th>Crystal C</th>
<th>Crystal D</th>
</tr>
</thead>
<tbody>
<tr>
<td>X-ray source (wavelength in Å)</td>
<td>EMBL, X11 (0.92)</td>
<td>Enraf-Nonius 4.5 kW (1.5418)</td>
<td>EMBL, X31 (1.4)</td>
<td>Enraf-Nonius 4.5 kW (1.5418)</td>
</tr>
<tr>
<td>Crystal dimensions (mm)</td>
<td>0.4, 0.2, 0.2</td>
<td>0.25, 0.12, 0.07</td>
<td>0.17, 0.17, 0.17</td>
<td>0.18, 0.18, 0.15</td>
</tr>
<tr>
<td>Data-collection temperature (K)</td>
<td>278</td>
<td>115</td>
<td>120</td>
<td>105</td>
</tr>
<tr>
<td>Mosaicity (°)</td>
<td>0.29±0.52</td>
<td>0.43±0.49</td>
<td>0.66±1.08</td>
<td>0.43±0.56</td>
</tr>
<tr>
<td>Unit-cell dimensions (Å)</td>
<td>98.24 (15), 101.25 (7), 90.80 (3)</td>
<td>98.54 (3), 98.89 (3), 90.62 (3)</td>
<td>98.58 (10), 98.82 (6), 90.62 (2)</td>
<td>98.68 (4), 98.72 (4), 90.66 (6)</td>
</tr>
<tr>
<td>Resolution (Å)</td>
<td>29.4–2.5</td>
<td>24.9–2.9</td>
<td>29.5–2.3</td>
<td>31.6–2.9</td>
</tr>
<tr>
<td>Total rotation scan (°)</td>
<td>95.0</td>
<td>94.2</td>
<td>137.2</td>
<td>217.7</td>
</tr>
<tr>
<td>Processed intensities</td>
<td>123925</td>
<td>76002</td>
<td>169486</td>
<td>175556</td>
</tr>
<tr>
<td>Unique intensities</td>
<td>33026</td>
<td>18768</td>
<td>37023</td>
<td>20265</td>
</tr>
<tr>
<td>Redundancy</td>
<td>3.5</td>
<td>4.9</td>
<td>4.6</td>
<td>8.7</td>
</tr>
<tr>
<td>Completeness (%)</td>
<td>99.6</td>
<td>92.8</td>
<td>92.5</td>
<td>99.9</td>
</tr>
<tr>
<td>$I/\sigma(I)$ (%)</td>
<td>81.3</td>
<td>83.7</td>
<td>90.3</td>
<td>97.7</td>
</tr>
<tr>
<td>$R_{int}$ (%)</td>
<td>17.5</td>
<td>10.9</td>
<td>13.7</td>
<td>24.0</td>
</tr>
<tr>
<td>$R_{merge}$ (%)</td>
<td>6.6</td>
<td>12.2 (42.2)</td>
<td>6.6 (24.8)</td>
<td>8.3 (10.4)</td>
</tr>
</tbody>
</table>

| (for putative space group $P4_2\overline{2}$) | |
| Completeness (%) (outer shell) | 96.9 | 93.1 | 83.8 | 99.9 |
| $I/\sigma(I)$ (%) (outer shell) | 4.3 | 3.0 | 5.1 | 9.7 |
| $R_{merge}$ (%) (outer shell) | 26.9 | 36.5 | 25.9 | 18.5 |
| Centric $[h^2 - l^2]$ zones 0kl, h0l, $h^2l$ | 1.002, 1.014, 0.999 | 0.905, 0.944, 0.869 | 0.753, 0.836, 0.788 | 0.764, 0.784, 0.739 |
| Acentric $[k^2 - l^2]$ || 0.759 | 0.690 | 0.603 | 0.573 |

† Outer shell (Å): crystal A, 2.49–2.45; crystal B, 2.85–2.90; crystal C, 2.34–2.30; crystal D, 2.94–2.89. ‡ The theoretical values for $|F| I$ in single crystals with a random distribution of atoms are 0.968 for centric reflections and 0.736 for acentric reflections.

3 μl of precipitant solution and 3 μl of protein solution (10 mg ml⁻¹) was deposited on a microbridge from DROP (Devis Realisation Outillage Precision, Grenoble) and equilibrated against precipitant solution consisting of 10% (v/v) PEG 6K buffered at pH 6.0 by Tris-maleic acid 100 mM, in a well of a Linbro box (Flow Laboratories, Inc., McLean, VA).

Cryo-stabilization of the crystals was only possible if performed in the cold room and in a very slow process. The well solution was replaced by the final cryo-protection solution, which consisted of the previously described precipitant solution complemented with glycerol to a final concentration of 25% (v/v). Precipitant solution was added to the sitting drops containing the crystals until a total volume of about 20 μl was reached. The cryo-protection solution was then added in minute amounts (ca. 0.2 μl) to the drops and was allowed to diffuse through the mother liquor and crystals. A faster increase in glycerol concentration invariably led to rupture of the crystals. The crystal cryo-stabilization proceeded over 2 d, until a final concentration of 25% glycerol was reached.

2.2. Crystallographic diffraction data collection and processing

Diffraction data from crystals grown at ambient or cold-room temperatures were obtained using an Enraf-Nonius FR570 rotating-anode generator operating at 4.5 kW with a Huber graphite monochromator to select Cu Kα radiation, or using synchrotron radiation at stations X31 and X11 at DESY, EMBL Outstation, Hamburg. Cryo-conditions were used for some of the data-collection experiments, using an Oxford Cryosystems Cryostream. Data were collected on image-plate devices from MAR Research and were processed with the DENZO/SCALEPACK (Otwinowski & Minor, 1997) suite of programs. The CCP4 package (Collaborative Computational Project, Number 4, 1994) was used to calculate observed structure factors and their intensity distribution (TRUNCATE), to normalize them (ECALC) and to calculate the self-rotation function (POLARRFN). SHELXS (Sheldrick, 1997) was used to calculate a twinning diagnostic parameter, $|F| I$. Table 1 indicates the data-collection conditions and statistics for the different diffraction data sets.

3. Results and discussion

3.1. Crystal data

ROO orange-brown crystals have a parallelepiped shape and have relative dimensions which vary from specimen to specimen. In favorable cases, they can reach 0.2–0.4 mm in their largest dimension within three weeks.

The crystals belong to space group $P2_12_12$, with unit-cell dimensions $a = 98.24\pm15$, $b = 101.25\pm7$, $c = 90.80\pm3$ Å for the best single-crystal data set collected thus far (crystal A in Table 1). When grown at room temperature, a gel-like precipitate develops during crystallization, which makes crystal handling difficult. On the other hand, if crystals are grown in the cold and/or with addition of detergent, the gel-like precipitate can be avoided and the number of crystals per drop controlled. The crystals’ shape and color remain essentially similar to those grown at room temperature, but their diffraction data show a Wilson cumulative distribution (Fig. 1) characteristic of merohedral twinning (Rees, 1980), with a recognizable sigmoidal shape below the exponential curve for the theoretical single-
For orthorhombic crystals, pseudo-mecro- 
dehedral twinning is possible in the case of 
degeneracy of the $a$ and the $b$ dimensions, 
which leads to a pseudo-tetragonal lattice. 
Additionally, an apparent symmetry 
increase in the intensity distribution from 
the orthorhombic to the tetragonal system 
will occur if the $ab$ diagonal is a twofold 
twinning axis. This will be fully accomplished 
in the case where the ratio between the two 
translators reaches 0.50.5. In fact, for all 
cold-room-grown ROO crystals, there 
was shrinkage of the $b$ axis towards the 
a-axis dimension, resulting in an ‘almost 
tetragonal’ lattice, with the twofold twinning 
axis lying along the $ab$ ‘diagonal’. The rela-
tive intensity distribution was then altered, 
and eventually (crystal $D$, last column in 
Table 1) displayed a pseudo-tetragonal 
$P4_2_2_1$ system. Details and statistics of the 
different diffraction data sets are given in 
Table 1.

3.2. Cryo-protection
The cryo-protection of ROO 
crystals was a delicate pro-
dure. Success could only be 
achieved when a very slow 
approach was tried in the cold 
room, as described in §2.1. If the 
addition of cryo-protectant was 
too fast, the crystals would 
typically start to fracture, 
beginning at the side with 
higher glycerol concentration. 
The diffraction properties 
(resolution and precision, as 
well as crystal stability) 
improved markedly by the use 
of cryo-techniques. At room 
temperature with a rotating-
anequeous source, data were not 
better than 3.5 Å, $R_{merge} = 13%$. 
(C. Frazão, data not shown). 
At 278 K and using synchrotron 
radiation, data were collected 
to 2.5 Å, $R_{merge} = 6.6%$ (crystal $A$ 
in Table 1). This compares 
favorably with data collected 
under cryo-conditions and with 
a rotating-anode source: 2.9 Å 
resolution, $R_{merge} = 12.2$ and 
8.3% (for crystals $B$ and $D$ 
in Table 1, respectively).

3.3. Pseudo symmetry
The calculated solvent 
content (Matthews, 1968) for 
the single orthorhombic crystals 
is 52%, assuming a protein 
homodimer in the asymmetric 
unit.
Self-rotation calculations, 
using normalized structure 
factors for the single-crystal 
diffraction data, indicated 
strong non-crystallographic 
symmetry features at the $\chi = 180^\circ$ 
and near the $\chi = 90^\circ$ sections (Figs. 2a and 2b). A 
strong peak at $\omega = 0.0, \varphi = 42.9, \chi = 180^\circ$ 
with an intensity height of 69% of the origin 
indicates a non-crystallographic twofold axis 
parallel to the pseudo-diagonal between 
axes $a$ and $b$ (their relative dimensions differ 
by only ca 3%). The association of this non-

crystallographic twofold rotation axis with 
the crystallographic orthorhombic symmetry 
produces an almost fourfold non-crystal-
lographic symmetry axis parallel to axis $c$ 
-detected at $\omega = 0.0, \varphi = 176.8$ or $12.7^\circ, \chi = 
85.8$ or $94.2^\circ$). The observed twinning in 
ROO crystals therefore seems reminiscent 
of the pseudo-symmetry within the asym-
metric unit.

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