Crystal Structure Analysis of Cytochrome c' by the Multiwavelength Anomalous Diffraction Method Using Synchrotron Radiation

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Abstract

A multiwavelength X-ray diffraction study was performed at 6 Å resolution on the native cytochrome c' crystal from Rhodospirillum rubrum, which contains heme irons. The X-ray intensity data from the crystal were collected on a four-circle diffractometer with three wavelengths, \( \lambda_1 = 1.077 \), \( \lambda_2 = 1.730 \) and \( \lambda_3 = 1.757 \) Å, using the synchrotron radiation produced by the storage ring in the Photon Factory, National Laboratory for High Energy Physics. \( \lambda_2 \) and \( \lambda_3 \) are above and below the energy of the K absorption edge of iron \( (\lambda_0 = 1.743 \, \text{Å}) \) respectively, while \( \lambda_1 \) is far from the edge. The positions of the two iron atoms in the crystal could be determined from the difference Patterson maps based on either the real or imaginary components of the anomalous scattering effect caused by iron. The best phase angles for the crystal were calculated from the X-ray intensity data measured with \( \lambda_1 \) and \( \lambda_2 \) by the method which is basically the same as the isomorphous replacement method. Although the resulting best phase angles differed 75° on the average from those obtained by the multiple isomorphous replacement method, the molecular boundary and α helices could be recognized on the electron density map.

1. Introduction

Since the first protein structure, sperm whale myoglobin, was determined by Kendrew, Dickerson, Strandberg, Hart, Davies, Phillips & Shore (1960), the isomorphous replacement method has been the most powerful tool for solving crystal structures of proteins, and most protein structures so far have been determined by this method. On the other hand, it has long been recognized that protein phase angles can be obtained from X-ray intensity data collected at several wavelengths (multiwavelength data) near an absorption edge of an anomalous scatterer which exists intrinsically in some proteins (Pepinsky & Okaya, 1956; Mitchell, 1957; Herzenberg & Lau, 1967; Phillips & Hodgson, 1980). This multiwavelength anomalous diffraction (MAD) method utilizes changes of scattering factor of an anomalous scatterer with wavelength. However, the MAD method has not been applied to protein crystallography, chiefly for lack of a tunable X-ray source.

The earliest work of structure analysis by the MAD method was performed by Hoppe & Jakubowski (1975). In their work, the X-ray intensity data were measured from an iron-containing protein, erythrocrucin, with Ni and Co Kα radiations. Recently, synchrotron radiation as an intense X-ray source with broad spectral distribution became available for protein crystallography, and experiments utilizing anomalous scattering effects have been initiated. The phase angles of rubredoxin, which is an iron-containing protein (one iron atom per 430 non-hydrogen atoms), were obtained by the use of film data recorded at several wavelengths near the iron K absorption edge (Phillips, Wlodawer, Goodfellow, Watenpaugh, Sieker, Jensen & Hodgson, 1977). The electron density map of the calcium-binding protein, parvalbumin, in which terbium was substituted for calcium (1.3 terbium atoms per 720 non-hydrogen atoms) in order to increase the anomalous scattering effect, was calculated by the MAD method using X-ray intensity data collected at three wavelengths near the terbium L₃ absorption edge (Kahn, FOURME, BOSSHARD, CHIADMI, RISLER, DIDEBERG & WERY, 1985). In this work, the electron density map of cytochrome c' was calculated by utilizing the anomalous scattering effect of iron. Although the molecular weight of cytochrome c' (one iron atom per 1000 non-hydrogen atoms) is larger than the two proteins mentioned above and the anomalous scattering effect of iron is smaller than that of terbium, an interpretable electron density map could be obtained by choosing wavelengths so as to give the maximum intensity difference owing to the real correction component of the anomalous scattering effect.

2. Data collection and processing

Cytochrome c' extracted from Rhodospirillum rubrum exists as a dimer that consists of identical polypeptide
chains each of 14,000 daltons molecular weight. The protoheme IX prosthetic group is covalently bound to cysteine residues of each polypeptide chain through thioether linkages. The crystals of cytochrome c' belong to the space group P61, with unit-cell dimensions a = b = 51.63 and c = 155.39 Å, and the asymmetric unit contains one dimer molecule (Yasui, Harada, Kai & Kasai, 1984).

X-ray diffraction experiments were performed using the X-ray beam produced by the storage ring in the Photon Factory, National Laboratory for High Energy Physics. A four-circle diffractometer located on the beam-line station 14A (BL-14A) was used for data collection (Satow, 1984). The diffractometer has a horizontal-type setup, and utilizes synchrotron radiation from a superconducting vertical wiggler (Huke & Yamakawa, 1980). The white X-ray beam, which has a higher degree of polarization in the vertical direction, is first monochromatized by an Si(111) double-crystal monochromator and then focused by a platinum-coated fused-quartz mirror (Satow, 1984). Multiwavelength data were collected at three wavelengths: λ1 = 1.077, λ2 = 1.730 and λ3 = 1.757 Å. Two crystals were used. The data sets collected from the first crystal at λ2 and λ3, which are on either side of the K absorption edge of iron (λK = 1.743 Å), contain Friedel intensities. λ1 is the wavelength near the L3 absorption edge of platinum where the anomalous scattering effect of iron is rather small. Data collection with λ1 was performed using the second crystal.

Ideally, X-rays whose wavelengths are similar to each other and are close to the iron K edge as possible would be better in order to avoid systematic error owing to absorption and in order to get a large anomalous scattering effect. Such a choice of wavelengths was employed in the case of rubredoxin (Phillips et al., 1977) and parvalbumin (Kahn et al., 1985). However, in this study wavelengths were chosen in a different way: (1) λ1 far from the absorption edge in order to obtain the maximum difference of f' from λ2 or λ3; (2) λ2 and λ3 slightly different from λK, since the actual bandwidth of a monochromatized incident X-ray beam may be broader than the energy resolution (ΔE/E) of the optics (2 × 10⁻⁴) owing to fluctuation of an electron orbit in the storage ring.

Calibrations of the wavelengths were performed by measuring absorption spectra of a lyophilized cytochrome c' in the iron K-edge region and K₂PtCl₆ powder in the platinum L3-edge region, respectively. As the intensity of the X-ray beam emitted from the storage ring decreased gradually during data collection, the intensity of the X-ray beam incident on the crystal was monitored by an ion chamber (Satow, 1984).

The raw data were corrected for Lorentz and polarization factors, radiation damage and absorption effects, using the program DRDCTN (Satow, unpublished work). The polarization factor was calculated assuming that the white X-ray beam incident to the monochromator was polarized 90% in the vertical direction. The absorption correction was based on the method of North, Phillips & Mathews (1968). Scaling factors among different data sets were calculated by Wilson's (1949) statistics. The conditions of data collection are summarized in Table 1.

### Table 1. Summary of the X-ray diffraction experiments

<table>
<thead>
<tr>
<th>Wavelength (Å)</th>
<th>λ₁ = 1.077</th>
<th>λ₂ = 1.730</th>
<th>λ₃ = 1.757</th>
</tr>
</thead>
<tbody>
<tr>
<td>f'</td>
<td>0.159</td>
<td>-4.493</td>
<td>-4.596</td>
</tr>
<tr>
<td>f''</td>
<td>1.775</td>
<td>3.891</td>
<td>0.474</td>
</tr>
<tr>
<td>d spacing (Å)</td>
<td>123-40</td>
<td>140-60</td>
<td>144-55</td>
</tr>
<tr>
<td>ω-scan speed (° s⁻¹)</td>
<td>40</td>
<td>40</td>
<td>40</td>
</tr>
<tr>
<td>R₁ ‡</td>
<td>0.040</td>
<td>0.019</td>
<td>0.046</td>
</tr>
<tr>
<td>ΔF real/F §</td>
<td>-</td>
<td>0.040</td>
<td>0.053</td>
</tr>
<tr>
<td>ΔF anom/F †</td>
<td>-</td>
<td>0.063</td>
<td>0.051</td>
</tr>
<tr>
<td>R₂/R₁</td>
<td>-</td>
<td>5.26</td>
<td>1.74</td>
</tr>
</tbody>
</table>

*The values of f' and f'' were taken from Sasaki (1984), which is based on Cromer & Liberman's (1970, 1981) method.
†R₂ = ∑[F(h) - F(h⁻¹)]²/∑F(h)², for equivalent reflections. R(h) is the mean of the measurements.
‡ΔF real/F = ∑[F(h) - F(h⁻¹)]²/∑F(h)².
§ΔF anom/F = ∑(F(h) - F(h⁻¹))/∑F(h) ± ∑(F(h) + F(h⁻¹)).

### 3. Results and discussion

#### 3.1. Data analysis

Fig. 1 shows a vector diagram illustrating the contributions from various parts of cytochrome c' to a structure factor. OA represents the contribution from the non-anomalous scattering atoms in the protein and AB corresponds to the normal structure factor from the two iron atoms. BC is the variation owing to the real correction component (f') of the anomalous scattering effect caused by iron. CD and CE are the variations owing to the imaginary correction component (f''). In this study, structure amplitudes F₁⁺, F₂⁺, F₁⁻, F₂⁻ and F₃ were measured for three wavelengths (Table 1), where F₁⁺ and F₁⁻ refer to hkl and hₚkl reflections measured with λ₁ (n = 1, 2 and 3). As the values of f'' at λ₁ and λ₃ are small (Table 1), the
following relations are presumed: \( F_1^+ = F_1^- = F_1 \) and \( F_3^+ = F_3^- = F_3 \). Furthermore, the values of \( |f'| \) at \( \lambda_2 \) and \( \lambda_3 \) are approximately equal to each other and are larger than that at \( \lambda_1 \) (Table 1). \( F_1, F_2, F_3 \) and \( F_3 \) are related to the vector diagram in Fig. 1 as follows: 

\[
E_1 = |OB|, \quad F_1^- = |OD|, \quad F_2^+ = |OE| \quad \text{and} \quad F_3 = |OC|.
\]

Furthermore, the average value of \( F_1^- \) and \( F_2^+ \), \( F_2^+ \), equals \( |OC| \) to a good approximation.

Table 2. Distribution of 458 reflections as a function of \( \Delta = |x_Fe - x_p| \)

<table>
<thead>
<tr>
<th>( \theta )</th>
<th>0°</th>
<th>30°</th>
<th>60°</th>
<th>90°</th>
<th>120°</th>
<th>150°</th>
<th>180°</th>
</tr>
</thead>
<tbody>
<tr>
<td>( a )</td>
<td>110</td>
<td>47</td>
<td>57</td>
<td>67</td>
<td>79</td>
<td>98</td>
<td></td>
</tr>
<tr>
<td>( b )</td>
<td>152</td>
<td>36</td>
<td>33</td>
<td>36</td>
<td>62</td>
<td>139</td>
<td></td>
</tr>
</tbody>
</table>

\( \lambda_2 \) (5.26) is large compared with that at \( \lambda_3 \) (1.74), indicating that the contribution of \( f" \) to the diffracted intensity is larger at \( \lambda_2 \) than at \( \lambda_3 \), as expected.

The high-noise peaks which appear in the same positions of Figs. 2(a) and (b) may be a result of systematic error caused by the absorption effect, because the absorption effect at \( \lambda_2 \) and \( \lambda_3 \) is larger than at \( \lambda_1 \).

Despite the fact that values of \( f' \) and \( f" \) are of the same order of magnitude as each other and that Friedel intensities were measured from the same crystal during the same beam time, the map for (c) is worst among the three difference Patterson maps (Fig. 2). Furthermore, the combined difference Patterson map with coefficient \( (F_1 - F_2)^2 + (k/2)^2(F_2^+ - F_2^-)^2 \), which must be a better estimate for iron than difference Patterson maps. The maps for (a) and (b) are based on the differences in \( f' \) for the two wavelengths and are analogous to the isomorphous difference Patterson map in the case of the isomorphous replacement method. The map for (c) is a regular anomalous difference Patterson map based on \( f" \) (Blundell & Johnson, 1976, pp. 337–362). Although the two Patterson maps at \( \theta = 1/2 \) with coefficients (a) and (b) (Fig. 2) were somewhat noisy and the highest peak did not coincide with the iron self vector, the positions of the two iron atoms could be determined by inspecting three Harker sections (\( \theta = 1/6, 1/3 \) and 1/2) and they were confirmed by checking iron cross vectors.

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\[ \Delta = |x_Fe - x_p| \]

\( x_p \) is calculated by (a) MIR and (b) MAD.

<table>
<thead>
<tr>
<th>( \theta )</th>
<th>0°</th>
<th>30°</th>
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<td>62</td>
<td>139</td>
<td></td>
</tr>
</tbody>
</table>

3.2. Location of the iron atoms

The positions of the two iron atoms in the crystal were determined from difference Patterson and difference Fourier maps. The coefficients (a) \( (F_1 - F_2)^2 \), (b) \( (F_1 - F_3)^2 \) and (c) \( (F_2 - F_3)^2 \) were used for the

![Fig. 2. Patterson maps of cytochrome c' (\( \theta = 1/2 \)) with coefficient (a) \( (F_1 - F_2)^2 \), (b) \( (F_1 - F_3)^2 \) and (c) \( (F_2 - F_3)^2 \). The positions of iron self vectors are marked. For the definitions of \( F_1, F_2, F_3 \) and \( F_3 \), see text.](image1)

![Fig. 3. Composites of four sections (\( \theta = 0.10-0.16 \)) of the difference Fourier maps of cytochrome c' with coefficient (a) \( (F_1 - F_3)^2 \), (b) \( (F_2 - F_3)^2 \) and (c) \( (F_2^+ - F_2^-)^2 \) using phase angles obtained by the MIR method (Yasui et al. 1985). The two iron positions (Fe1 and Fe2) are indicated.](image2)
was similar to the map for (a). These observations may be explained by the relation between $\alpha_{Fe}$ (phase angle of iron) and $\alpha_p$ (phase angle of protein). Distribution of $|\alpha_{Fe} - \alpha_p|$ for 458 reflections up to 6 Å resolution tends to 0 or $\pi$ rather than random chance (Table 2), suggesting that the map for (c) is not a good estimate for iron (Blundell & Johnson, 1976, pp. 337–362).

Difference Fourier maps were calculated with the coefficients (a) $(F_1 - F_2)\exp(i\alpha)$, (b) $(F_1 - F_3)\exp(ia)$ and (c) $(F_1^* - F_2^*)\exp[i(\alpha - \pi/2)]$, where $\alpha$ is the protein phase angle obtained by the multi-isomorphous replacement (MIR) method (Yasui, Harada, Kai & Kasai, 1985) (Fig. 3). Each difference Fourier map revealed two large peaks at the expected positions of the two iron atoms.

As $\alpha$ is very small at $\lambda_3$ (Table 1), Patterson and Fourier maps based on the difference between $F_1^*$ and $F_2^*$ were featureless and did not give the positions of the two iron atoms.

### 3.3. Phase calculation

As Fig. 1 shows, a change in $f'$ with wavelength produces a change in X-ray diffraction pattern; intensity data collected with different wavelengths can be used to calculate phase angles in the same way as the isomorphous replacement method, if positions of atoms which cause anomalous scattering effects are known. The best phase angles of cytochrome c' were calculated by the MAD method using the X-ray intensity data collected with $\lambda_1$ and $\lambda_2$. Although $f'$ at $\lambda_3$ is close to that at $\lambda_2$, $f''$ at $\lambda_3$ is very small (Table 1); the intensity data collected with $\lambda_3$ were not included in the phase calculation. Parameters of the two iron atoms were refined by minimizing

$$[F_2 - F_1 \exp(i\alpha_{best}) + f]^2$$

where $f$ is the $f'$ contribution of the two iron atoms at $\lambda_2$ and $\alpha_{best}$ is the best phase angle calculated by the MAD method. [For details of the phase calculation, see Blundell & Johnson (1976, pp. 363–380), Hendrickson & Teeter (1981) and Phillips & Hodgson (1980).] The resulting best phase angles were compared with those obtained by the isomorphous replacement method and the average phase discrepancy for the 462 reflections up to 6 Å resolution was 75°. Phillips et al. (1977) reported that phases for the $hk0$ reflections of rubredoxin calculated by the MAD method differed by approximately 60° (on the average) from the phases of the refined model. Kahn et al. (1985) compared MAD phases of the Tb-substituted parvalbumin with phases calculated from a model and got an average phase discrepancy of about 54°. Fig. 4(b) shows the electron density map calculated by the MAD method. Although the electron density map is noisy compared with that calculated by the isomorphous replacement method (Fig. 4a), $\alpha$ helices and the molecular boundary can be recognized.

### 4. Concluding remarks

In this study, location of the heme irons and calculation of the phase angles were successfully performed for cytochrome c' by using the multiwavelength data collected at three wavelengths ($\lambda_1$, $\lambda_2$ and $\lambda_3$). The method of phase calculation was basically the same as that of single isomorphous replacement combined with the anomalous scattering effect. As it seems that the actual bandwidth of an X-ray beam depends on the stability of the orbit of an electron in the storage ring rather than the energy resolution of the optics ($\Delta E \sim 1.4$ eV at $\lambda_3$), wavelengths which were sufficiently far from $\lambda_3$ by about 55 eV were used for data collection. The improvement in the stability of the synchrotron radiation will allow a more delicate choice of wavelengths and, consequently, accurate phase determination from multiwavelength data.

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### References


