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Crystallization and preliminary X-ray diffraction analysis of cytochrome *c′* from *Rubrivivax gelatinosus* at 1.3 Å resolution

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Abstract

Cytochrome *c′* from the purple non-sulfur phototrophic bacterium *Rubrivivax gelatinosus* has been crystallized by vapour diffusion at pH 5, 6.3 and 8, in sodium acetate, sodium citrate, and Tris–HCl buffers, respectively. Crystals grown at pH 5 and 6.3 diffract, respectively, to 2.0 Å (298 K) and 1.4 Å (100 K) using synchrotron radiation. Data up to 1.3 Å resolution with 99.8% completeness were collected at 100 K on a crystal grown at pH 8. The space group is $P3_121$ or $P3_221$, and the unit-cell parameters are $a = b = 69.63$, $c = 123.63$ Å.

1. Introduction

Cytochromes *c′* are widely found in phototrophic (Bartsch, 1978; Meyer & Kamen, 1982), denitrifying (Iwasaki *et al.*, 1991), nitrogen-fixing (Yamanaka & Imai, 1972), and sulfur-oxidizing (Schmidt & DiSpirito, 1990) bacteria. Although their physiological function is still unknown, a role in electron transfer has been suggested. The X-ray structures of cytochrome *c′* from *Rhodospirillum molischianum* (Finzel *et al.*, 1985, Weber *et al.*, 1981), *Rhodospirillum rubrum* (Yasui *et al.*, 1992), *Chromatium vinosum* (Ren *et al.*, 1993), *Alcaligenes denitrificans* (Baker *et al.*, 1995), *Achromobacter xylosoxidans* (Baker *et al.*, 1995), and *Rhodobacter capsulatus* (Tahirov *et al.*, 1996) reveal that cytochromes *c′* are characterized by a four-helix bundle structural motif, by covalent attachment of the haem to a C*XX*CH motif, and by the presence of a pentacoordinated Fe ion. The latter is bound axially to a solvent-exposed His residue, with an empty coordination site, tightly packed by the surrounding aromatic and hydrophobic amino acid residues, pointing toward the protein core.

Cytochromes *c′* have peculiar spectroscopic and magnetic properties (Moore & Pettigrew, 1990) and, in particular, the spin state of the ferric cytochrome *c′* has been the subject of controversy. Optical (Horio & Kamen, 1961), magnetic susceptibility (Ehrenberg & Kamen, 1965; Tasaki *et al.*, 1967), near infrared (Kamen *et al.*, 1973), near infrared MCD (Rawlings *et al.*, 1977), Mössbauer (Emptage *et al.*, 1977), and NMR (Banci *et al.*, 1992,; Bertini *et al.*, 1990, Bertini *et al.*, 1993; Emptage *et al.*, 1981, Jackson *et al.*, 1983; La Mar *et al.*, 1981, La Mar *et al.*; 1990) studies have suggested that cytochrome *c′* is substantially high-spin ($S = 5/2$; $^6A_1$) at neutral pH. In contrast, EPR (Fujii *et al.*, 1995; Maltempo, 1974, 1975; Maltempo *et al.*, 1974; Maltempo & Moss, 1976; Monkara *et al.*, 1992; Yoshimura *et al.*, 1987, 1990), Mössbauer (Maltempo *et al.*, 1980), optical (Maltempo, 1976), NMR (La Mar *et al.*, 1990) and resonance Raman (Strekas & Spiro, 1974) results have been interpreted with the hypothesis of a quantum mechanical admixture of a high spin ($S = 5/2$; $^6A_1$) and an intermediate spin ($S = 3/2$; $^4A_2$) state at neutral pH.

In all cytochromes *c′* so far investigated, the contribution of the intermediate spin state to the ground state appears to decrease as the pH increases, resulting in a high-spin ferric ion at pH 11.0 (Fujii *et al.*, 1995). NMR spectroscopic studies, carried out in order to elucidate the molecular basis for this pH-modulated transition (Banci *et al.*, 1992; Bertini *et al.*, 1990, 1993; Emptage *et al.*, 1981; Jackson *et al.*, 1983; La Mar *et al.*, 1990), have revealed the presence of a large spectral shift in the 7–10 pH range, attributed to deprotonation of the Nδ1 of the proximal histidine ligand. It is generally accepted that the spin state variability reflects the strength of the axial ligand crystal field. In particular, a small iron displacement from the mean haem plane toward a weakly coordinated protonated axial His residue is thought to be correlated with a larger contribution of the intermediate spin state. This hypothesis is confirmed in model porphyrin complexes, where there is a clear correlation between spin state of the haem iron and the Fe displacement from the plane (Fujii *et al.*, 1995), but it has not been proved in proteins, probably because of the poor resolution of the X-ray structures available for cytochromes *c′*.

In an attempt to provide strong structural support for the correlation between spin state, Fe displacement, and ionization state of amino-acid residues in the vicinity of the haem in

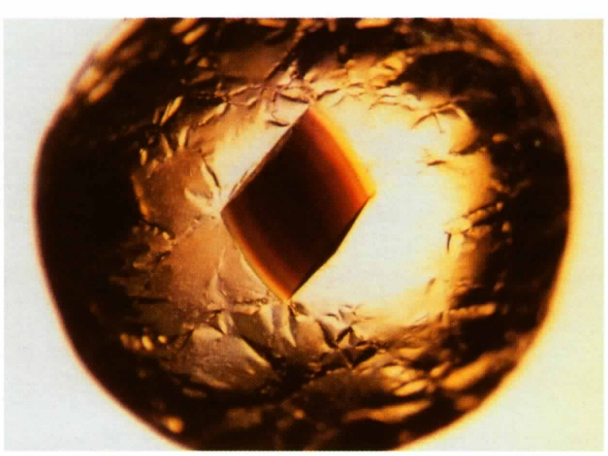

Fig. 1. A single crystal (2.0 × 1.8 × 0.8 mm) of cytochrome *c′* from *R. gelatinosus* obtained using 100 m*M* Tris–HCl, pH 8, containing 2.5 *M* $(NH_4)_2SO_4$.

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cytochromes *c′*, we have undertaken a focused study of the structure of a single cytochrome *c′* as a function of pH. In this work we report the crystallization of cytochrome *c′* from the purple phototrophic bacterium *Rubrivivax gelatinosus* at pH = 5, 6.3 and 8, together with data collection and preliminary diffraction analysis to 1.3 Å resolution, obtained using cryogenic conditions and synchrotron radiation.

2. Materials and methods

2.1. *Protein purification and crystallization*

R. gelatinosus (DSM, Deutsche Sammlung von Mikroorganismen, Göttingen, Germany, type strain 1709) was grown at 298 K under photoheterotrophic conditions (medium 27, DSM). Cytochrome *c′* was isolated and purified in the oxidized state following the procedure described by Bartsch (Bartsch, 1971). Purity was checked by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE).

Crystallization trials on the freshly purified cytochrome were performed at 293 K by the hanging-drop method, using 5 ml of a 20 mg ml^{-1} protein solution in 20 m*M* Tris–HCl, pH = 8, and diluting this volume with 5 ml of precipitant solution. The drop was equilibrated by vapour diffusion against 1 ml of precipitant solution using an Hampton Research 24-well Linbro plate.

The initial screening for determination of the crystallization conditions was carried out using 100 m*M* sodium acetate (pH 4 and 5), 100 m*M* sodium citrate (pH 6.3 and 7), or 100 m*M* Tris–HCl (pH 8 and 8.5) buffers containing 1.6–3.2 *M* $(NH_4)_2SO_4$. Formation of crystalline material was observed only using 2.4–2.6 *M* ammonium sulfate. In particular, large regular trigonal prisms grew to an average size of about 1.6 × 1.4 × 0.6 mm after 3 d using 100 m*M* Tris–HCl, pH 8, containing 2.5 *M* ammonium sulfate, whereas large crystals 0.8 × 0.8 × 0.6 mm on average grew after 3 d using 100 m*M* sodium citrate, pH 5, containing 2.5 *M* ammonium sulfate. Using 100 m*M* sodium citrate (pH 7) or 100 m*M* Tris–HCl (pH 8) in the presence of 30%(*w*/*v*) polyethyleneglycol (PEG) 6000 (Sigma) as precipitant, formation of rhombohedral twinned platelets was observed after 3 d, whereas when 1 *M* LiCl was used together with PEG as co-precipitant in 100 m*M* sodium citrate (pH 6.3) large regular trigonal prisms 1.2 × 1.0 × 0.8 mm grew after 5 d. In all cases a single crystal formed in each drop. A large variety of crystallization conditions, in particular spanning a wide range of pH units, were thus suitable for successful crystallization.

2.2. *Crystallographic diffraction data collection and evaluation*

A single crystal of cytochrome *c′* (Fig. 1) with dimensions 2.0 × 1.8 × 0.8 mm, grown using 100 m*M* Tris–HCl, pH 8 containing 2.5 *M* ammonium sulfate as precipitant solution, was transferred from the mother liquor to the cryobuffer (20% glycerol in the precipitant solution). After ∼1 min, the crystal was scooped up in a rayon cryoloop, and rapidly exposed to a cold nitrogen stream (Oxford Cryosystems Cryostream) on the X11 beamline (λ = 0.9077 Å) of the DORIS storage ring at the EMBL outstation at the Deutsches Elektronen Synchrotron (DESY) in Hamburg (Germany). Diffraction data were collected at 100 K using a 30 cm MAR Research imaging plate scanner (Hamburg, Germany). A single crystal was used to record the entire data set in three sweeps, at different exposure times, in order to record accurately both the strongest low-resolution, and the weakest high-resolution diffraction intensities.

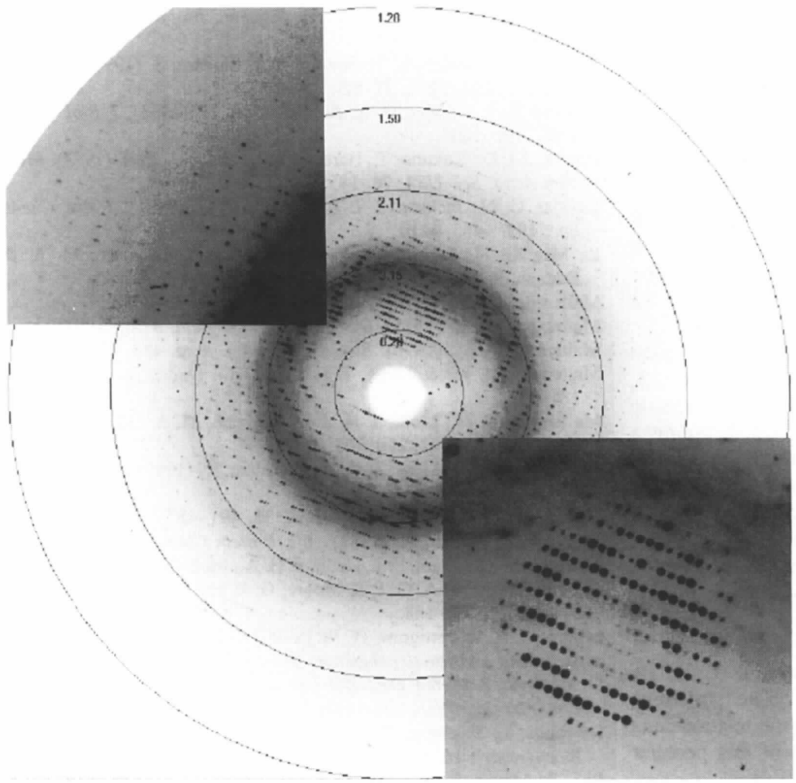

Fig. 2. Diffraction pattern from an oscillation image of cytochrome *c′* from *R. gelatinosus*. Resolution markers are indicated. Insets show details of the image at higher magnification. The upper, left inset shows the diffraction at the high-resolution limit.

Table 1. *Summary of X-ray data collection on R. gelatinosus cytochrome c′ at pH 8*

The numbers in parentheses relate to the highest resolution bin (1.31–1.29 Å).

Space group	$P3_121$ or $P3_221$
Unit-cell parameters (Å)	
a	69.63
b	69.63
c	123.63
Beamline at EMBL	X11
Temperature (K)	100
Lowest resolution (Å)	20
Maximum resolution (Å)	1.3
Wavelength (Å)	0.9077
Raw measurements used	838578
Unique reflections	87744
% Completeness	99.8 (99.3)
% greater than 3σ	80.7
I/σ in highest resolution bin	3.58

The images were processed with *DENZO* and merged with *SCALEPACK* (Otwinowski, 1988), resulting in data from 20 to 1.3 Å resolution and 99.8% completeness. Table 1 reports a summary of data-collection procedures, data statistics, and the results of data analysis for *R. gelatinosus* cytochrome *c′*.

3. Results and discussion

3.1. *Data collection and evaluation*

Crystals of cytochrome *c′* obtained using acetate buffer at pH 5 and ammonium sulfate as precipitant diffract well in the synchrotron beam, reaching a resolution of 2.0 Å at room temperature. In order to increase the quality and resolution of the data, X-ray diffraction data were collected under cryogenic (100 K) conditions (Hope, 1990; Rogers, 1994; Watenpaugh, 1991). Crystals grown in citrate buffer at pH 6.3, using PEG and LiCl as precipitant, diffract to a resolution of 1.4 Å, using 5% glycerol in the precipitant solution as cryoprotectant. However, the highest resolution (1.3 Å) was achieved with crystals grown using Tris–HCl buffer at pH 8, containing 2.5 *M* ammonium sulfate as precipitant. The quality of the diffraction thus obtained is evident in the image shown in Fig. 2 and in the data summary given in Table 1.

R. gelatinosus cytochrome *c′* crystallizes in the trigonal space group $P3_121$ or $P3_221$, with unit-cell dimensions $a = b = 69.63$, $c = 123.63$ Å. Assuming one dimer (28 kDa) per asymmetric unit, the volume-to-mass ratio, V_m, is 3.1 Å^3 Da^{-1}, giving a solvent content of 60.2%. These values are in the range found for proteins (Matthews, 1968). The structure of *A. xylosoxidans* (Baker *et al.*, 1995) (PDB code: 1CGO) will be used as a search model for structure solution using molecular replacement, because of the high homology (52%) of its sequence (Ambler, 1973) with *R. gelatinosus* cytochrome *c′* (Ambler & Meyer, 1979).

The preliminary results obtained on crystals grown at different pH values suggest that atomic resolution could be attainable for *R. gelatinosus* cytochrome *c′*. The structures should provide a clear basis for understanding the relationships between structural and spectroscopic features of this peculiar class of cytochromes.

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