

Miki Senda,^a Shinya Kishigami,^b
Shigenobu Kimura^c and Toshiya
Senda^{d*}

^aJapan Biological Information Research Center (JBIRC), Japan Biological Informatics Consortium (JBIC), 2-42 Aomi, Koto-ku, Tokyo 135-0064, Japan, ^bGraduate School of Life Science, University of Hyogo, 3-2-1 Kouto, Kamigori, Hyogo 678-1297, Japan, ^cDepartment of Biomolecular Functional Engineering, University of Ibaraki, 4-12-1 Nakanarusawa, Hitachi, Ibaraki 316-8511, Japan, and ^dBiological Information Research Center (BIRC), National Institute of Advanced Industrial Science and Technology (AIST), 2-42 Aomi, Koto-ku, Tokyo 135-0064, Japan

Correspondence e-mail: tsenda@jbirc.aist.go.jp

Received 2 April 2007

Accepted 10 May 2007

Crystallization and preliminary X-ray analysis of the electron-transfer complex of Rieske-type [2Fe–2S] ferredoxin and NADH-dependent ferredoxin reductase derived from *Acidovorax* sp. strain KKS102

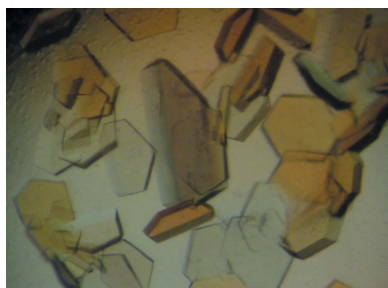
The electron-transfer complex of BphA3, a Rieske-type [2Fe–2S] ferredoxin, and BphA4, a NADH-dependent ferredoxin reductase, was crystallized using the sitting-drop vapour-diffusion method under anaerobic conditions. The obtained crystals were analyzed by SDS–PAGE, which showed that they contained both BphA3 and BphA4. The crystals belong to space group $P2_1$, with unit-cell parameters $a = 60.60$, $b = 173.72$, $c = 60.98$ Å, $\beta = 115.8^\circ$, and diffracted to a resolution of 1.9 Å.

1. Introduction

BphA3 and BphA4 comprise an electron-transfer system for the multi-component dioxygenase BphA, which is derived from the PCB-degrading bacterium *Acidovorax* sp. (formally *Pseudomonas* sp.) strain KKS102 (Kimbara *et al.*, 1989). BphA3 (MW 11.7 kDa, 109 amino acids) and BphA4 (MW 44 kDa, 408 amino acids) are a Rieske-type [2Fe–2S] ferredoxin and an FAD-containing NADH-dependent ferredoxin reductase, respectively (Kikuchi *et al.*, 1994). BphA4 receives two electrons from NADH as a form of hydride and transfers an electron to each of two BphA3 molecules. BphA3 shuttles between BphA4 and the BphA1A2 complex to transport an electron. The BphA1A2 complex receives two electrons from two BphA3 molecules. These electrons are used for the activation of an oxygen molecule in the catalytic reaction. In order to elucidate the molecular mechanism of the electron transfer between BphA3 and BphA4, the crystal structure of BphA4 was determined by our group (Senda *et al.*, 2000), showing that this protein has a similar fold to that of glutathione reductase. On the basis of the crystal structure, the BphA3-binding site and the residues involved in the electron transfer have been predicted (Senda *et al.*, 2000).

For further analysis of the electron-transfer mechanism between BphA3 and BphA4, crystal structure analysis of the BphA3–BphA4 complex is indispensable. However, BphA3 and BphA4 cannot form a stable complex when both are in their oxidized form. It is therefore difficult to obtain a nonproductive complex under aerobic conditions, as previous examples of the complex between plant-type ferredoxin and ferredoxin reductase have shown (Morales *et al.*, 2000; Müller *et al.*, 2000; Kurisu *et al.*, 2001). Since BphA3 and BphA4 seem to form a complex in a redox-dependent manner, reduced-form BphA4 should be mixed with oxidized BphA3 under anaerobic conditions to prepare the stable complex between them. Thus, anaerobic crystallization is essential to crystallize the productive complex between BphA3 and BphA4.

For anaerobic crystallization, we developed an anaerobic chamber system (Senda *et al.*, 2007), which we successfully used to isolate BphA3 in the reduced form and then to crystallize it (Senda *et al.*, 2007). This system is suitable for the crystallization of the redox-dependent BphA3–BphA4 complexes. In the present study, we



crystallized the productive complex by mixing NADH-reduced BphA4 and oxidized BphA3 under anaerobic conditions. Since the previously obtained crystal structures of the ferredoxin–ferredoxin reductase complexes were of nonproductive electron-transfer complexes (Morales *et al.*, 2000; Müller *et al.*, 2000; Kurisu *et al.*, 2001), which were obtained by mixing ferredoxin and ferredoxin reductase both in the oxidized forms, the present crystal is the first example of a productive complex between ferredoxin and ferredoxin reductase. Thus, the crystal structure of the present complex would enable in-depth insights to be obtained into the functional implications of the electron-transfer complex. Here, we report the anaerobic crystallization of the electron-transfer complex between BphA3 and BphA4 and its preliminary crystallographic analysis.

2. Methods and results

2.1. Protein purification

BphA3 was overexpressed in *Escherichia coli* BL21 cells containing expression plasmid pCA3 and purified under aerobic conditions as described previously (Kimura *et al.*, 2005). Briefly, the cells were cultured at 310 K for 30 h after induction with 0.2 mM isopropyl β -D-thiogalactopyranoside (IPTG). The synthesized BphA3 was purified from the cells by anion-exchange and gel-filtration chromatography. The purified BphA3 was in the oxidized form, as confirmed by its absorption spectrum, which showed characteristic peaks at 460 and 590 nm (Fig. 1a; Kimura *et al.*, 2005). The BphA3 was concentrated to 17.5 mg ml⁻¹ in 25 mM potassium phosphate buffer pH 7.0 containing 0.15 M sodium chloride, 1 mM dithiothreitol and 2% (w/v) glycerol. This BphA3 solution was used for crystallization.

BphA4 was overexpressed and purified under aerobic conditions using a modification of the previously published procedure (Yamada *et al.*, 2000). *E. coli* JM109 cells containing expression plasmid pKH204 were grown in LB medium containing 50 μ g ml⁻¹ ampicillin. The cells were cultured at 310 K. Expression of BphA4 was induced with 0.2 mM IPTG at an OD₆₀₀ of 0.3–0.4. After 8 h induction, the cells were harvested and disrupted in TE buffer (10 mM Tris–HCl, 1 mM EDTA pH 7.4) containing 2 mM phenylmethanesulfonyl-fluoride (PMSF) using a French press in order to obtain a cell extract. Cell debris was removed by centrifugation at 12 000 rev min⁻¹ for 30 min. The supernatant was diluted 2.5 times in buffer A (10 mM potassium phosphate buffer, 1 mM EDTA pH 7.0) and then applied onto an anion-exchange column (Whatman; DE52, 26 \times 120 mm)

equilibrated with buffer A. BphA4 was eluted using a linear gradient of 10–100 mM potassium phosphate buffer pH 7.0 containing 1 mM EDTA. The fractions containing BphA4 were combined and buffer-exchanged in buffer A using an Amicon Ultra-15 (Millipore) with a molecular-weight cutoff of 30 kDa. The BphA4 solution was concentrated to about 10 mg ml⁻¹. The concentrated BphA4 solution was applied onto a Blue-Sepharose 6 Fast Flow column (GE Healthcare Biosciences; 16 \times 120 mm) equilibrated with buffer A. BphA4 was eluted using a linear 0–0.5 M NaCl gradient in buffer A. The obtained BphA4 fractions were combined and buffer-exchanged in buffer A using an Amicon Ultra-15 (Millipore) with a molecular-weight cutoff of 30 kDa. The purified BphA4 was in the oxidized form, as confirmed by its absorption spectrum, which showed characteristic peaks at 375 and 450 nm (Yamada *et al.*, 2000). The obtained BphA4 was concentrated to 26.4 mg ml⁻¹ in 50 mM potassium phosphate buffer pH 7.0. This BphA4 solution was used for crystallization.

2.2. Crystallization

The BphA3–BphA4 complex was crystallized under anaerobic conditions. Anaerobic conditions were prepared using an Anaerobox ‘HARD’ anaerobic chamber (Hirasawa) filled with a gas mixture (96% N₂, 4% H₂; Senda *et al.*, 2007). The anaerobic condition was checked with a BR0055B anaerobic indicator (Oxoid) prior to the experiment. The set-up of our anaerobic chamber has been described in detail previously (Senda *et al.*, 2007). Concentrated BphA3 and BphA4 were brought into the anaerobic chamber and subjected to gas exchange in order to remove dissolved oxygen. The O₂ in the protein solutions was removed by pipetting for 1–3 min every 1 h for 4 h. The anaerobic conditions of the resultant protein solutions were analyzed using a BR0055B anaerobic indicator; in most cases the indicator showed no O₂ contamination in the protein solutions.

The first step in preparing the protein solution for crystallization was the reduction of BphA4 using excess NADH. BphA4 at a concentration of 26.4 mg ml⁻¹ (0.6 mM) in 50 mM potassium phosphate buffer pH 7.0 was reduced with 20 mM NADH (from a stock solution of 100 mM NADH) for 5 min at 277 K. The colour of the solution changed from yellow to blue, suggesting the formation of the semiquinone (one-electron reduced) form. The absorption spectrum, however, showed the characteristic peaks of the semiquinone form at 575 and 617 nm and of the hydroquinone (two-electron reduced) form at 731 nm (Fig. 1b), suggesting that the BphA4 solution contained both the semiquinone and hydroquinone forms. In this

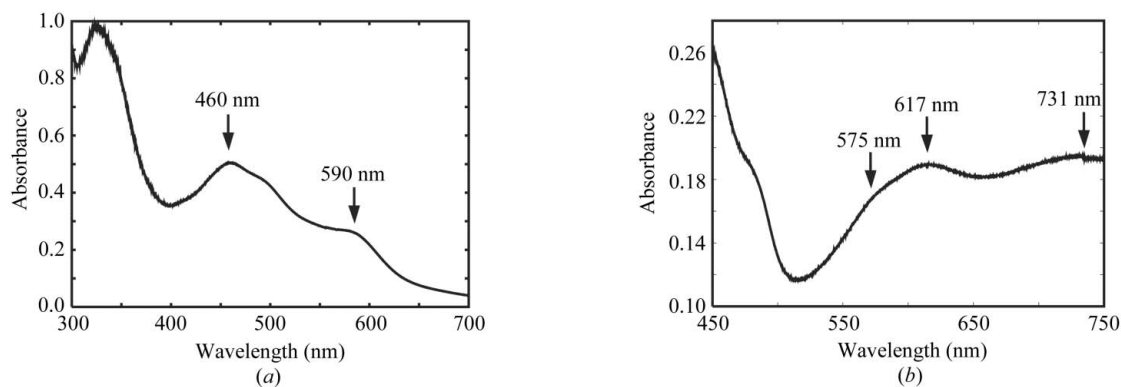


Figure 1 Absorption spectra of BphA3 and BphA4 before crystallization. (a) Absorption spectrum of BphA3 used in the crystallization experiment. The spectrum shows that BphA3 is in the oxidized form. (b) Absorption spectrum of BphA4 used in the crystallization experiment. The spectrum shows that the BphA4 solution contained both the semiquinone and hydroquinone forms.

procedure, anaerobic BphA4 and NADH solutions were mixed in a plastic tube that had been stored under aerobic conditions. The oxygen molecules adsorbed on the plastic tube seemed to be used for partial one-electron oxidation of hydroquinone BphA4.

Next, we prepared the protein mixture using reduced BphA4 and oxidized BphA3. 137.5 μl reduced BphA4 at a concentration of 21.1 mg ml^{-1} (0.48 mM) was mixed with 52.8 μl oxidized BphA3 at a concentration of 17.5 mg ml^{-1} (1.5 mM) and incubated for 5 min at 277 K. The resultant protein solution was used for crystallization screening. The concentrations of BphA3 (420 μM) and BphA4 (350 μM) in the solution are more than 20 times higher than the K_d value. Details of the K_d -value analysis will be published elsewhere. Initial crystal screening was performed by the sitting-drop vapour-diffusion method using Crystal Screens I and II (Hampton Research) and Wizard I and II (Emerald Biosystems) at 293 K. Reservoir solutions were degassed as described previously (Senda *et al.*, 2007). A sitting drop was prepared by mixing 0.9 μl each of the protein and reservoir solutions and was equilibrated against 500 μl reservoir solution. Thin plate crystals appeared in 3 d in Crystal Screen I condition No. 9 [0.2 M ammonium acetate, 0.1 M sodium citrate pH 5.6, 30% (w/v) polyethylene glycol 4000] at 293 K. A larger crystal was obtained using a larger droplet, mixing 4.0 μl each of protein and reservoir solutions at 293 K. Plate-shaped crystals (approximately $0.7 \times 0.5 \times 0.05 \text{ mm}$) were obtained in 1–2 weeks (Fig. 2). The crystals obtained were analyzed by SDS-PAGE. The crystals were washed three times in the artificial mother liquor, dissolved in distilled water and loaded onto an SDS-PAGE gel. The SDS-PAGE results showed that the crystals contained both BphA3 and BphA4 (Fig. 3). The molecular weights of the additional bands *a* and *b* found in the higher molecular-weight region (Fig. 3) correspond to those of the BphA4 dimer and the BphA3–BphA4 complex, respectively. A densitometry analysis of the gel using the program *ImageJ* (Abramoff *et al.*, 2004) suggested that the molar ratio of BphA3 to BphA4 is approximately 1:1 in the crystal (Fig. 3). Crystals could not be obtained using the same protein and reservoir solutions under aerobic conditions, suggesting that anaerobic conditions are essential for obtaining the crystal of the BphA3–BphA4 complex.

2.3. X-ray data collection and processing

The crystals were frozen using liquid nitrogen in the anaerobic chamber (Anaerobic 'HARD', Hirasawa). Prior to freezing the crystals, the crystals were cryoprotected by adding 10 μl reservoir solution to the droplet. After 2–3 min, the crystals were flash-cooled

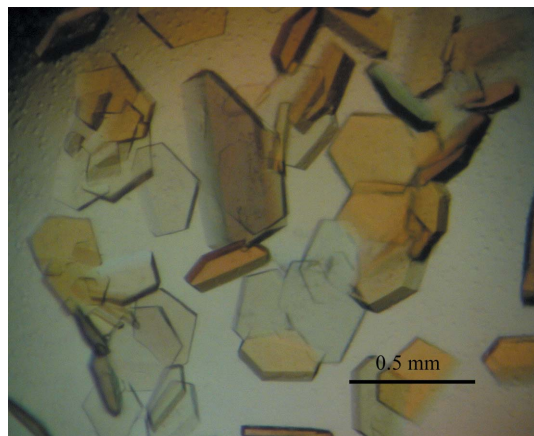


Figure 2
Crystals of the BphA3–BphA4 complex obtained under anaerobic conditions.

in liquid nitrogen. The frozen crystals were stored in a dry shipper (SC 4/3V, MVE) to transport them to the synchrotron facility for data collection. Diffraction data were collected at 100 K using an ADSC Quantum 210 CCD detector at beamline NW12A of Photon Factory-AR (PF-AR; Tsukuba, Japan; Fig. 4). The diffraction data were processed and scaled using the programs *XDS* and *XSCALE*

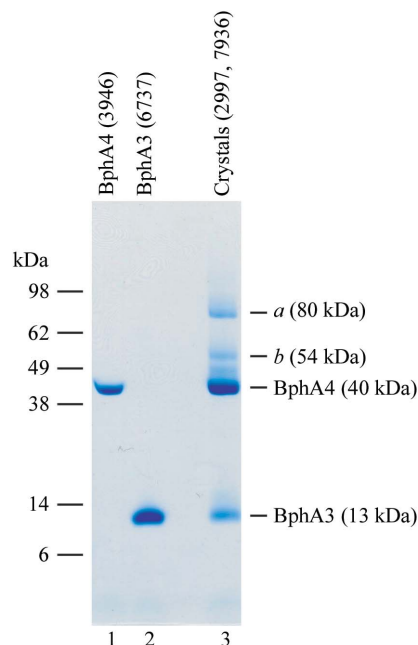


Figure 3
SDS-PAGE of BphA3–BphA4 crystals stained with Coomassie Brilliant Blue. The estimated molecular weight of each band is given in parentheses. Band densities are given in parentheses above the gel. 25.7 pmol BphA4 and 96.6 pmol BphA3 were loaded on lanes 1 and 2, respectively. Taken into account the band densities on the gel, BphA3 and BphA4 on lane 3 correspond to 43 and 48 pmol, respectively.

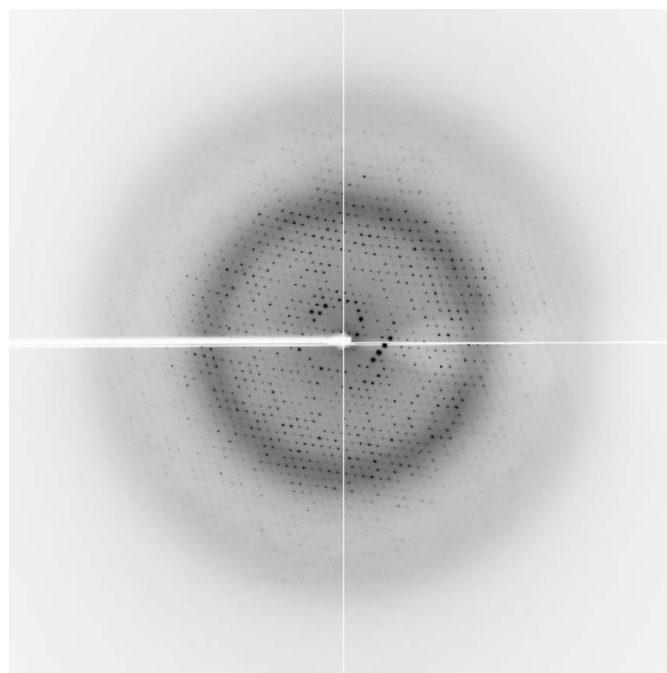


Figure 4
X-ray diffraction pattern of the BphA3–BphA4 complex crystal. The diffraction data were collected at beamline NW12A of PF-AR using an ADSC Quantum 210 CCD detector.

Table 1

Data-collection statistics.

Values in parentheses are for the outermost resolution shell.

	High-resolution data	Low-resolution data
Beamline	NW12A (PF-AR)	NW12A (PF-AR)
Crystal-to-detector distance (mm)	140	190
Oscillation angle (°)	0.5	0.5
Exposure time (s)	8	1
Wavelength (Å)	0.9780	0.9780
Space group	$P2_1$	
Unit-cell parameters (Å, °)	$a = 60.60$, $b = 173.72$, $c = 60.98$, $\beta = 115.8$	
Resolution limit (Å)	18–1.9 (2.00–1.90)	
Observations	799740	
Unique reflections	86196	
Completeness (%)	96.7 (93.3)	
$I/\sigma(I)$	21.2 (5.5)	
Redundancy	9.3 (6.8)	
R_{merge}	0.082 (0.484)	
Mosaicity (°)	0.32	

(Kabsch, 1993). The diffraction data at high and low resolution were collected separately from one crystal, as many low-resolution diffractions were saturated by 8 s exposures. The two data sets were merged using the program *XSCALE* (Table 1). The crystal belongs to space group $P2_1$, with unit-cell parameters $a = 60.60$, $b = 173.72$, $c = 60.98$ Å, $\beta = 115.8^\circ$. Assuming the presence of one BphA3–BphA4 complex comprised of one BphA4 homodimer and two BphA3 monomers in the asymmetric unit, the Matthews coefficient (V_M ; Matthews, 1968) was calculated to be $2.6 \text{ Å}^3 \text{ Da}^{-1}$, which corresponds to a solvent content of 52.2%. A typical diffraction

pattern is shown in Fig. 4. The data-collection statistics are given in Table 1. Molecular-replacement calculations are in progress.

We thank Professor Fukuda for continuous support of our work. This study was supported in part by the New Energy and Industrial Technology Development Organization (NEDO) of Japan.

References

- Abramoff, M. D., Magelhaes, P. J. & Ram, S. J. (2004). *Biophotonics Int.* **11**, 36–42.
- Kabsch, W. (1993). *J. Appl. Cryst.* **26**, 795–800.
- Kikuchi, Y., Nagata, Y., Hinata, M., Kimbara, K., Fukuda, M., Yano, K. & Takagi, M. (1994). *J. Bacteriol.* **176**, 1689–1694.
- Kimbara, K., Hashimoto, T., Fukuda, M., Koana, T., Takagi, M., Oishi, M. & Yano, K. (1989). *J. Bacteriol.* **171**, 2740–2747.
- Kimura, S., Kikuchi, A., Senda, T., Shiro, Y. & Fukuda, M. (2005). *Biochem. J.* **388**, 869–878.
- Kurisu, G., Kusunoki, M., Katoh, E., Yamazaki, T., Teshima, K., Onda, Y., Ariga, Y. K. & Hase, T. (2001). *Nature Struct. Biol.* **8**, 117–121.
- Matthews, B. W. (1968). *J. Mol. Biol.* **33**, 491–497.
- Morales, R., Kachalova, G., Vellieux, F., Charon, M. H. & Frey, M. (2000). *EMBO Rep.* **1**, 271–276.
- Müller, J. J., Lapko, A., Bourenkov, G., Ruckpaul, K. & Heinemann, U. (2000). *J. Biol. Chem.* **276**, 2786–2789.
- Senda, M., Kishigami, S., Kimura, S. & Senda, T. (2007). *Acta Cryst.* **F63**, 311–314.
- Senda, T., Yamada, T., Sakurai, N., Kubota, M., Nishizaki, T., Masai, E., Fukuda, M. & Mitsui, M. (2000). *J. Mol. Biol.* **304**, 397–410.
- Yamada, T., Sakurai, N., Nishizaki, T., Senda, T., Masai, E., Fukuda, M. & Mitsui, Y. (2000). *Protein Pept. Lett.* **7**, 277–280.