New molecular packing in a crystal of pseudoazurin from *Alcaligenes faecalis*: a double-helical arrangement of blue copper

Yohta Fukuda, Eiichi Mizohata and Tsuyoshi Inoue*

Department of Applied Chemistry, Graduate School of Engineering, Osaka University, 2-1 Yamadaoka, Suita, Osaka 565-0871, Japan. *Correspondence e-mail: inouet@chem.eng.osaka-u.ac.jp

Received 6 February 2017

Accepted 15 February 2017

Edited by Z. Dauter, Argonne National Laboratory, USA

Keywords: pseudoazurin; blue copper protein; macromolecular crowding effect; molecular packing; *Alcaligenes faecalis*.

PDB reference: pseudoazurin, 5x31

Supporting information: this article has supporting information at journals.iucr.org/f

Pseudoazurin from the denitrifying bacterium *Alcaligenes faecalis* (AfPAz) is a blue copper protein and functions as an electron donor to copper-containing nitrite reductase (CuNIR). Conventionally, AfPAz has been crystallized using highly concentrated ammonium sulfate as a precipitant. Here, a needle-like crystal of AfPAz grown in a solution containing a macromolecular precipitant, polyethylene glycol 8000 (PEG 8000), is reported. The crystal belonged to space group $P6_1$, with unit-cell parameters $a = b = 68.7$, $c = 94.2$ Å. The structure has been determined and refined at 2.6 Å resolution. The asymmetric unit contained two AfPAz molecules contacting each other on negatively charged surfaces. The molecular packing of the crystal showed a right-handed double-helical arrangement of AfPAz molecules and hence of blue copper sites. This structure provides insight into the excluded-volume effect of PEG and the manner of assembly of AfPAz.

1. Introduction

Crystallization is one of the most important steps in protein crystallography (McPherson & Gavira, 2014). Although it has more than a 150-year history, there is no royal road to growing diffraction-quality protein crystals and hence it remains a thorn in the protein crystallographer's side. Once one crystallization condition for a protein has been discovered, few researchers search for different conditions unless they are guaranteed to provide new biological information such as ligand-binding information, structural differences at various pH values or complex structures with other proteins. However, exploring different crystallization conditions is important to establish a guideline for the rational design of protein molecular packings, because protein crystals have been regarded as novel practical frameworks for biomolecular devices (Abe & Ueno, 2015; McGovern *et al.*, 2015). One of the model proteins for protein crystal engineering is the blue copper protein, which functions in electron-transport chains and contains a mononuclear copper (Cu) centre that is usually coordinated by two histidine (His) residues, one cysteine (Cys) residue and one methionine (Met) residue. The reason this copper site is called blue copper is that it shows a bluish or greenish colour owing to strong absorption of visible light at around 600 nm (and 450 nm in the case of some proteins) originating from ligand-to-metal charge transfer between the thiolate S atom of Cys and the oxidized Cu atom (Solomon *et al.*, 2004). To date, several factors that can manipulate the molecular packing of a blue copper protein are known from studies on plastocyanin (PCy): molecular bridging by metal ions, the presence of polyethylene glycol (PEG) in the crystallization solution

Table 1

Macromolecule-production information.

Source organism	<i>A. faecalis</i> S-6
Expression vector	pET-24c
Expression host	<i>E. coli</i> BL21 (DE3)
Complete amino-acid sequence of the construct produced†	MASENIEVHMLNKGAGAMVFEPAYIKANPGDVT- TFIPVDKGHNVESIKDMIPEGAKEFKSKINEN- YVLTVTQPGAYLVKCTPHYAMGMIALIAVGDS- PANLDQIVSAKKPKIVQERLEKVIASAK

† The initial three residues (underlined) are additional residues owing to insertion into the vector.

(Crowley *et al.*, 2008, 2009) and chemical modification of the protein with PEG (Cattani *et al.*, 2015).

Pseudoazurin (PAz) is a blue copper protein which typically consists of eight β -strands and two α -helices (Adman, 2006). The blue copper site in PAz has a distorted tetrahedral geometry showing a highly anisotropic electron paramagnetic resonance spectrum, which is a mixture of axial and rhombic signals (Abdelhamid *et al.*, 2007; Yamaguchi *et al.*, 2016). As of January 2017, structures of PAzes from *Alcaligenes faecalis* (Petratos *et al.*, 1987, 1988), *Achromobacter cycloclastes* (Inoue *et al.*, 1993), *Methylobacterium extorquens* (Inoue *et al.*, 1994), *Paracoccus pantotrophus* (Williams *et al.*, 1995), *Hyphomicrobium denitrificans* (Hira *et al.*, 2009a) and *Shinorhizobium meliloti* (Laming *et al.*, 2012) have been reported. PAz from *A. faecalis* (AfPAz) was the first PAz for which the structure was determined via X-ray crystallography (Petratos *et al.*, 1987), and more than a third of the PAz structures deposited in the Protein Data Bank (PDB) are of PAz from *A. faecalis*. It mediates electron transport to dissimilatory copper-containing nitrite reductase (CuNIR; Kakutani *et al.*, 1981), structures of which have also been determined by X-ray crystallography (Murphy *et al.*, 1997a). The electron-transfer (ET) complex between AfPAz and CuNIR from *A. faecalis* (AfNIR) has been studied using a nuclear magnetic resonance technique (Impagliazzo *et al.*, 2007; Vlasie *et al.*, 2008), but its crystal structure has never been determined. Meanwhile, preliminary crystallographic data on ET complexes between PAz and CuNIR from *A. cycloclastes* (AcPAz and AcNIR; Murphy *et al.*, 1997b) and *H. denitrificans* (HdPAz and HdNIR; Hira *et al.*, 2009b) have been reported. Also, a xenogeneic ET complex structure between HdPAz and CuNIR from *Achromobacter xylosoxidans* (AxNIR) has recently been solved by Nojiri and coworkers (Nojiri, 2016). AfPAz crystal structures have been determined in various states: oxidized and reduced states (Libeu *et al.*, 1997), reduced forms at different pH values (Vakoufari *et al.*, 1994), amino-acid substitution mutants (Libeu *et al.*, 1997), metal-free (Petratos *et al.*, 1995) and metal-substituted forms (Gessmann *et al.*, 2011, 2015), and a chemically modified protein (Prudêncio *et al.*, 2004). All of these crystals, however, belong to the same space group, $P6_5$, with similar unit-cell parameters ($a = b \simeq 50$, $c \simeq 90$ Å), presumably because the crystallization solution always contained ammonium sulfate. Here, we report a new AfPAz crystal grown in a polyethylene glycol (PEG) solution, which was found while seeking crystallization conditions for the ET complex between AfPAz

and AfNIR. Our data provide insight into the excluded-volume effect of PEG in protein crystallization and the assembly of blue copper proteins, which are expected to be applied to bio-electronic devices (Maruccio *et al.*, 2005).

2. Materials and methods

2.1. Macromolecule production

Recombinant AfPAz was expressed in *Escherichia coli* BL21 (DE3) cells using the expression vector pET-24c (MacPherson *et al.*, 2010), which was a kind present from Professor Michael E. P. Murphy at the University of British Columbia. Transformed *E. coli* cells were cultivated in Luria–Bertani medium at 310 K to an OD₆₀₀ of 0.6, and 0.5 mM isopropyl β -D-1-thiogalactopyranoside (IPTG) was added to induce expression. The culture was grown for a further 6 h at 310 K. The cells were collected by centrifugation (8983g for 15 min), resuspended in 20 mM Tris–HCl pH 8.0 and sonicated using a SONICSTAR 85 ultrasonic homogenizer (AS ONE). After the soluble fraction had been separated by centrifugation (20 000g for 1 h), 20 mM Tris–HCl buffer pH 8.0 containing 10 mM CuSO₄ was gradually dropped into the sample at room temperature to generate holo copper-binding sites. Excess CuSO₄ was eliminated by iterative dialysis to 20 mM Tris–HCl buffer pH 8.0. AfPAz was purified at 277 K using a 5 ml HiTrap Q column (GE Healthcare) and a HiLoad 16/60 Superdex 75 pg column (GE Healthcare). The purity of the sample was checked by SDS–PAGE. The purified sample was concentrated to 40 mg ml^{−1} and stored at 193 K. Macromolecule-production information is shown in Table 1.

2.2. Crystallization

AfPAz crystals with a new appearance were obtained in the course of searching for crystallization conditions for the ET complex between AfPAz and AfNIR. The initial screening was performed with a Mosquito crystallization robot (TTP Labtech) and the Crystal Screen, PEG Rx, PEG/Ion and Salt Rx screening kits from Hampton Research. The crystallization method in this step was sitting-drop vapour diffusion at 293 K. 0.1 μ l protein solution containing 10 mg ml^{−1} AfPAz and 5 mg ml^{−1} AfNIR, which was purified as described previously (Fukuda, Tse, Nakane *et al.*, 2016), was mixed with 0.1 μ l reservoir solution on VIOLAMO 96-well plates (AS ONE). Greenish-blue needle-like crystals appeared using Crystal Screen condition No. 28 [0.2 M sodium acetate trihydrate, 0.1 M sodium cacodylate trihydrate pH 6.5, 30% (w/v) polyethylene glycol 8000 (PEG 8000)] in a month. We then performed a second screening using the hanging-drop vapour-diffusion method and various concentrations of PEG 8000 because the obtained crystals were too fine and small to use in X-ray diffraction experiments. The reservoir solution (400 μ l) was poured into 0.5 ml sample cups (Sanplatec) and 1.5 μ l protein solution (10 mg ml^{−1} AfPAz and 5 mg ml^{−1} AfNIR) was mixed with 1.5 μ l reservoir solution on siliconized cover glass plates. Diffraction-quality crystals were grown within a week with a reservoir solution consisting of 0.2 M sodium

Table 2
Crystallization condition.

Method	Hanging-drop vapour diffusion
Plate type	0.5 ml sample cup (14 mm diameter × 24.6 mm) and siliconized cover glass
Temperature (K)	293
Protein concentration	10 mg ml ⁻¹ AfPAz and 5 mg ml ⁻¹ AfNIR (estimated from absorption at 280 nm)
Buffer composition of protein solution	20 mM Tris-HCl pH 8.0
Composition of reservoir solution	0.2 M sodium acetate trihydrate, 0.1 M sodium cacodylate trihydrate pH 6.5, 32.5–37.5% (w/v) PEG 8000
Volume and ratio of drop	1.5 µl:1.5 µl (protein solution:reservoir)
Volume of reservoir (µl)	400

Table 3
Data collection and processing.

Values in parentheses are for the outer shell.

Diffraction source	BL44XU, SPring-8
Wavelength (Å)	0.90000
Temperature (K)	100
Detector	MX300-HE, Rayonix
Beam size (µm)	70.0 × 70.0 [h × w]
Attenuator	0.6 mm Al
Crystal-to-detector distance (mm)	307
Rotation range per image (°)	1
Total rotation range (°)	62
Exposure time per image (s)	1
Space group	<i>P</i> 6 ₁
<i>a</i> , <i>b</i> , <i>c</i> (Å)	68.7, 68.7, 94.2
α , β , γ (°)	90, 90, 120
Mosaicity (°)	0.24
Resolution range (Å)	50.0–2.50 (2.54–2.50)
Total no. of reflections	32454
No. of unique reflections	8741
Completeness (%)	99.2 (99.8)
Multiplicity	3.7 (3.9)
$\langle I/\sigma(I) \rangle$	13.4 (6.1)
<i>R</i> _{int} (%)	14.5 (27.9)
CC _{1/2} /CC*†	(0.953)/(0.988)
Overall <i>B</i> factor from Wilson plot (Å ²)	24.4

† CC_{1/2} and CC* are the Pearson correlation coefficient of two half data sets and an estimate of the true CC value, respectively. CC* is calculated using the following equation: CC* = [2CC_{1/2}/(1 + CC_{1/2})]^{1/2}.

acetate trihydrate, 0.1 M sodium cacodylate trihydrate pH 6.5, 32.5–37.5% (w/v) PEG 8000 (Fig. 1). A crystal for X-ray data collection was harvested using a 18 mm Mounted CryoLoop with a loop diameter of 0.3–0.4 mm (Hampton Research), flash-cooled in a cryostream (93 K) and stored in liquid nitrogen. We did not need to transfer the crystal to a cryo-

Table 4
Structure solution and refinement.

Values in parentheses are for the outer shell.

PDB code	5x31
Bragg spacings (Å)	36.9–2.60 (2.67–2.60)
Completeness (%)	99.3 (99.7)
No. of reflections used in refinement	7369 (534)
Final <i>R</i> _{work}	0.196 (0.245)
Final <i>R</i> _{free}	0.239 (0.279)
Cruickshank DPI (Å)	0.311
No. of non-H atoms	
Protein	1896
Cu	2
Water	32
Total	1930
R.m.s. deviations	
Bonds (Å)	0.028
Angles (°)	1.96
Average <i>B</i> factors (Å ²)	
Overall	24.5
Protein	24.5
Cu	20.0
Water	22.4
Ramachandran plot	
Most favoured (%)	97.2
Allowed (%)	2.8
Disallowed (%)	0

protectant solution owing to the high PEG concentration in the crystallization condition. Crystallization details are summarized in Table 2. Pure AfPAz (10 mg ml⁻¹) without AfNIR could be crystallized in the same condition and the crystal showed the same needle-like appearance (Supplementary Fig. S1).

2.3. Data collection, processing, structure solution and refinement

X-ray diffraction intensity data were collected on the BL44XU beamline at SPring-8, Hyogo, Japan at cryogenic temperature (100 K) using an MX300-HE charge-coupled device (CCD) detector (Rayonix) (Table 3). The data set was indexed, integrated and scaled using *HKL-2000* (Otwinowski & Minor, 1997). Although the total scanning range for data collection was 180° with 1° rotation per image, only the first 62 images were used for data processing; the latter 118 images were rejected since they significantly degraded the quality of the data. Owing to the small size of the measured crystal (approximately 5 × 5 × 300 µm), X-ray radiation damage may

Figure 1
Crystals of AfPAz formed under (a) a PEG condition and (b) a salt condition (Kakutani *et al.*, 1981; Petratos *et al.*, 1987).

be rapidly accumulated. The phases were determined by the molecular-replacement (MR) method using *MOLREP* (Vagin & Teplyakov, 2010) with an *AfPAz* molecule (PDB entry 1paz; Petratos *et al.*, 1988) as a search model. The resolution range used for MR was 10–3.0 Å. Manual model building using a resolution range of 36.9–2.60 Å was performed using *Coot* (Emsley *et al.*, 2010). *REFMAC5* (Murshudov *et al.*, 2011) as implemented in the *CCP4* suite (Winn *et al.*, 2011) and *phenix.refine* from the *PHENIX* suite (Adams *et al.*, 2010) were used for structural refinement. The final models were checked for stereochemical quality using *PROCHECK* (Laskowski *et al.*, 1993) and *MolProbity* (Chen *et al.*, 2010). Data-collection and processing statistics are summarized in Table 3.

3. Results and discussion

As of January 2017, there are 31 crystal structures of PAz or its mutants in the PDB. Salts were used as precipitants for 28 PAz

structures and *AfPAz* has previously only been crystallized under ammonium sulfate conditions. The new *AfPAz* crystal reported here, however, grew in a solution containing PEG 8000 as a macromolecular precipitant, and showed a needle-like appearance that differed from that of known *AfPAz* crystals (Fig. 1). The greenish-blue colour of the crystal showed that the Cu sites were oxidized. This crystal could belong to space group $P6_1$ or $P6_5$; therefore, scaling was performed using both space groups. Because a unique solution showing an R factor of 0.387 and a final CC of 0.617 was obtained only when we used data indexed in space group $P6_1$, we adopted these data for structure refinement. Although reflections to 2.5 Å resolution were included for scaling based on a high $CC_{1/2}$ in the highest resolution shell (0.95) as well as the high $\langle I/\sigma(I) \rangle$ value of 6.1 (Table 3), the following refinement step revealed that using data to 2.5 Å resolution and default external restraint weights in *REFMAC5* increased the gap between R_{work} and R_{free} to more than 7%, while a

Figure 2

Molecular packing of *AfPAz* crystals. (a, b, c) The lattice of the crystal grown with PEG 8000 (space group $P6_1$, present work). Molecules A and B are shown in blue and yellow, respectively. Cu atoms are drawn as spheres. Symmetry-related molecules are illustrated with pale colours. Green lines are the edges of the unit cell. (d, e, f) The lattice of the crystals grown with ammonium sulfate (space group $P6_5$, previous work; PDB entry 8paz). PAz molecules are shown in green. Symmetry-related molecules are illustrated in pale green. Black lines are the edges of the unit cell. Figures were prepared using PyMOL (DeLano, 2002).

resolution cutoff at 2.6 Å resolution kept it smaller than 5%. We therefore rejected reflections higher than 2.6 Å resolution in refinement. The final model gave R_{work} and R_{free} values of 19.6 and 23.9%, respectively. Refinement statistics are summarized in Table 4. Probably because of the limiting size of the crystal used (Fig. 1*a*) and accumulated radiation damage, we could not collect high-resolution data. However, a new crystallographic technique using an X-ray free-electron laser, serial femtosecond crystallography, will overcome this problem because high-resolution structures of copper-containing proteins without radiation damage can be obtained using microcrystals (Fukuda, Tse, Nakane *et al.*, 2016; Fukuda, Tse, Suzuki *et al.* 2016).

The asymmetric unit in our crystal contained two AfPAz molecules (molecules *A* and *B*) as described in Figs. 2(*a*), 2(*b*) and 2(*c*), while one AfPAz molecule was found in the asymmetric unit of the previously reported AfPAz crystals (Figs. 2*d*, 2*e* and 2*f*). The two AfPAz molecules in the asymmetric unit in our crystal had a pseudo-twofold axis between them and showed a C^α root-mean-square deviation (r.m.s.d.) value of 0.22 Å. No electron density derived from AfNIR was

observed and there were no vacant spaces that could accommodate AfNIR molecules in the molecular packing in our crystal, even though it was grown in a solution containing AfNIR. The C^α r.m.s.d. values between our structure and the previous one in the oxidized state (PDB entry 8paz; Libeu *et al.*, 1997) were 0.68 Å for molecule *A* and 0.72 Å for molecule *B*. These low C^α r.m.s.d. values indicate that the difference in molecular packing was not caused by structural changes of the AfPAz molecules. Because both our crystal and the crystal used to obtain PDB entry 8paz were grown at pH 6.5, there were small differences in the protonation states of the side chains, which can affect the manner of molecular packing. Moreover, additional N-terminal residues originating from the vector sequence (Table 1) are not the reason for the new packing, because our sample could be packed in the previously reported crystal form (Fig. 1*b*). One of the possible factors that can change the molecular packing is the macromolecular-crowding effect. There were two concentrated macromolecules in our crystallization condition: PEG 8000 and AfNIR. Because the same crystal form was obtained without AfNIR (Supplementary Fig. S1), it is reasonable to infer that PEG 8000 mainly affected the molecular packing. The facts that the solvent content in our crystal was ~48%, which was lower than the values of ~54% in other AfPAz crystals, and the Matthews coefficient (V_M) of our crystal was 2.3 Å³ Da⁻¹, which is lower than the value of 2.7 Å³ Da⁻¹ for other AfPAz crystals, are consistent with the report that the presence of PEG 8000 in the crystallization solution makes the molecular packing of PCy tighter because of the excluded-volume effect of PEG, which enhances protein–protein interaction through loosely packed low-specificity interfaces (Crowley *et al.*, 2009). Our structure supports such a macromolecular-crowding effect of PEG, because molecules *A* and *B* symmetrically interacted with each other on the negatively charged hence electrostatically repulsive surface (~264 Å²) where β -strands 4 and 6 are located (red square in Fig. 3*a*). The corresponding surface is contacted by a positively charged surface including a 3₁₀-helix located near the head of AfPAz in the previous packing of crystals grown under salt conditions (blue square in Fig. 3*b*). The negatively charged surface in our structure also interacted with the positively charged surface containing the 3₁₀-helix (blue square in Fig. 3*a*); that is, symmetry-related molecules interact with each other using electrostatic attraction. The same position in the previous packing is occupied by a less charged surface containing α -helix 1 (grey square in Fig. 3*b*). These facts provide a presumable crystallization scenario for our crystal: negatively charged surfaces of two AfPAz molecules (*A* and *B* mentioned above) primarily come close to each other in the presence of high-concentrated macromolecules and then stack up along the *c* axis through electrostatic interaction, resulting in the needle-like crystal.

Another notable characteristic of our AfPAz crystal is that molecules *A* and *B* formed a helix and assembled a right-handed double helix along the *c* axis (Fig. 2*a*). Interestingly, when PCy is modified by PEG 5000, the PEGylated protein forms a double-helical assembly in the crystal (Cattani *et al.*, 2015). The high concentration of PEG contained in our

Figure 3

Molecular packings and electrostatic potential surfaces of AfPAz ranging from positive (blue) to negative (red). Blue and red squares indicate interfaces with positively and negatively charged surfaces of neighbouring molecules, respectively. Blue and red characters show positively and negatively charged secondary structures in neighbouring molecules, respectively. A grey square indicates an interface with a less charged surface of a neighbouring molecule. Grey characters show a less charged secondary structure in a neighbouring molecule. Cu ions are shown by spheres. (*a*) Molecular packing of the crystal presented here. Molecules *A* and *B* are coloured blue and yellow, respectively. (*b*) Molecular packing of the previously reported crystal structure (PDB entry 8paz). PAz molecules are coloured green. The electrostatic surface potential was calculated by APBS (Baker *et al.*, 2001) and the figure was prepared using CueMol (<http://cuemol.sourceforge.jp/en/>).

crystallization condition may be relevant to a double-helical assembly, as PEGylation accelerates the formation of right-handed double-helix assemblies of PCy. Although many blue copper proteins often make a head-to-head 'dimer' in the molecular packing and this 'dimer' is thought to be relevant to intermolecular ET (van Amsterdam *et al.*, 2002; Sato *et al.*, 2005), PAz does not seem to prefer such an interaction in crystals. There are only two exceptions: *HdPAz* (Hira *et al.*, 2009a) and engineered *AcPAz* with the ligand loop structure of amicyanin (Velarde *et al.*, 2007). These were reported to be packed in a head-to-head manner. The head-to-head contact may not be favourable for the self-interaction of native PAzes because they have lysine residues which form a positively charged ring surrounding a hydrophobic head (Adman, 2006). Furthermore, negatively charged residues located at the other end of the protein can accelerate a head-to-tail or other types of interaction. Therefore, the mean distance from one Cu atom to the closest different Cu atom in the molecular packings of all reported Cu-containing PAzes is 27 Å. The minimum and maximum are 10.9 Å (head-to-head *HdPAz*) and 33.6 Å (reduced P80A mutant of *AfPAz*; Libeu *et al.*, 1997), respectively. The mean shortest Cu–Cu distance in non-head-to-head packings is 30 Å, which is significantly longer than the 10.9–12.9 Å found in head-to-head PAz 'dimers' (Velarde *et al.*, 2007; Hira *et al.*, 2009a). Because our *AfPAz* crystal did not show a head-to-head packing, the Cu–Cu distance in the asymmetric unit ($\text{Cu}^{\text{A}}\text{--Cu}^{\text{B}}$) was 34.4 Å. However, the distances between a Cu atom and a symmetry-related Cu atom in a neighbouring asymmetric unit (Cu_{asym}) were 19.2 Å ($\text{Cu}^{\text{A}}\text{--Cu}_{\text{asym}}^{\text{A}}$) and 19.3 Å ($\text{Cu}^{\text{B}}\text{--Cu}_{\text{asym}}^{\text{B}}$), respec-

tively (Fig. 4b). The regularly spaced Cu-atom arrangement constituted a right-handed double helix along the *c* axis (Figs. 2b, 2c and 4). The Cu atoms in the PEGylated PCy crystal are also equally spaced, as found in our structure; however, the shortest Cu–Cu distance in the helical PCy assembly is about 35 Å, because PEGylated PCy is packed in a head-to-tail manner. The macromolecular-crowding effect is thought to make PCy form oligomers which are involved in electron hopping through neighbouring molecules (Sato *et al.*, 2005). Molecular packings of PCy crystallized under concentrated PEG 8000 conditions, which mimic the crowding in the cell (Minton, 2006), show a copper arrangement in which symmetry-related Cu atoms are close enough to each other (<25 Å) to transfer electrons and these assemblies are thought to imitate a physiological intermediate structure in electron tunnelling (Crowley *et al.*, 2008). The electron-hopping mechanism through the PCy assembly is a relatively new idea compared with the conventional model that the displacement of a single PCy molecule is important for efficient ET. Our present crystal was also crystallized in a highly concentrated macromolecule condition and showed short Cu–Cu distances in symmetry-related molecules; therefore, the observed molecular packing may be relevant to the biological functions of *AfPAz*.

Protein self-assemblies have been enthusiastically studied because they have potential for novel applications such as catalysts (Margolin & Navia, 2001), bio-electronics (Atanasova *et al.*, 2011; Lee *et al.*, 2009; Maruccio *et al.*, 2005) and medical materials (Cormode *et al.*, 2010). Protein crystals are one of the best frameworks for these purposes. For example, a

Figure 4

Right-handed double helix composed of blue copper sites. Cu atoms of molecules A and B are coloured blue and yellow, respectively. (a) Stereoview. (b) Cu distances shown in Å. This figure was prepared using *PyMOL*.

zinc porphyrin-substituted myoglobin crystal containing oxo-centred triruthenium clusters was recently developed as an efficient photoinduced ET system (Koshiyama *et al.*, 2011). The assembly of a blue copper protein and electron hopping through it also have caught attention in the area of bio-electronics because it can be exploited in a protein-based field-effect transistor (Maruccio *et al.*, 2005). The Cu–Cu_{asym} distances in our crystal are short enough for long-range ET through the protein matrix (Tezcan *et al.*, 2001; Crane *et al.*, 2001). Therefore, our AfPAz crystal may provide useful information for the design of blue copper protein-based electronic devices.

Acknowledgements

This work was supported by a Grant-in-Aid for Research Activity startup from the Japan Society for the Promotion of Science (grant No. 16H06940 to YF). The authors thank Professor Dr A. Nakagawa, Dr E. Yamashita and Dr A. Higashiura for their support in data collection on BL44XU at SPring-8 (proposal No. 2016B6644) and members of the Inoue laboratory for their comments on this study. YF prepared the crystal and analysed the data, EM performed data collection and YF and TI wrote the manuscript. The authors declare no competing financial interests.

References

- Abdelhamid, R. F., Obara, Y., Uchida, Y., Kohzuma, T., Dooley, D. M., Brown, D. E. & Hori, H. (2007). *J. Biol. Inorg. Chem.* **12**, 165–173.
- Abe, S. & Ueno, T. (2015). *RSC Adv.* **5**, 21366–21375.
- Adams, P. D. *et al.* (2010). *Acta Cryst.* **D66**, 213–221.
- Adman, E. T. (2006). In *Handbook of Metalloproteins*, edited by A. Messerschmidt, R. Huber, K. Wieghardt & T. Poulos. Chichester: John Wiley & Sons. <https://doi.org/10.1002/0470028637.met186>.
- Amsterdam, I. M. C. van, Ubbink, M., Einsle, O., Messerschmidt, A., Merli, A., Cavazzini, D., Rossi, G. L. & Canters, G. W. (2002). *Nature Struct. Biol.* **9**, 48–52.
- Atanasova, P., Rothenstein, D., Schneider, J. J., Hoffmann, R. C., Dilfer, S., Eiben, S., Wege, C., Jeske, H. & Bill, J. (2011). *Adv. Mater.* **23**, 4918–4922.
- Baker, N. A., Sept, D., Joseph, S., Holst, M. J. & McCammon, J. A. (2001). *Proc. Natl Acad. Sci. USA*, **98**, 10037–10041.
- Cattani, G., Vogeley, L. & Crowley, P. B. (2015). *Nature Chem.* **7**, 823–828.
- Chen, V. B., Arendall, W. B., Headd, J. J., Keedy, D. A., Immormino, R. M., Kapral, G. J., Murray, L. W., Richardson, J. S. & Richardson, D. C. (2010). *Acta Cryst.* **D66**, 12–21.
- Cormode, D. P., Jarzyna, P. A., Mulder, W. J. & Fayad, Z. A. (2010). *Adv. Drug Deliv. Rev.* **62**, 329–338.
- Crane, B. R., Di Bilio, A. J., Winkler, J. R. & Gray, H. B. (2001). *J. Am. Chem. Soc.* **123**, 11623–11631.
- Crowley, P. B., Matias, P. M., Khan, A. R., Roessle, M. & Svergun, D. I. (2009). *Chemistry*, **15**, 12672–12680.
- Crowley, P. B., Matias, P. M., Mi, H., Firbank, S. J., Banfield, M. J. & Dennison, C. (2008). *Biochemistry*, **47**, 6583–6589.
- DeLano, W. L. (2002). *PyMOL*. <http://www.pymol.org>.
- Emsley, P., Lohkamp, B., Scott, W. G. & Cowtan, K. (2010). *Acta Cryst.* **D66**, 486–501.
- Fukuda, Y., Tse, K. M., Nakane, T. *et al.* (2016). *Proc. Natl Acad. Sci. USA*, **113**, 2928–2933.
- Fukuda, Y., Tse, K. M., Suzuki, M. *et al.* (2016). *J. Biochem.* **159**, 527–538.
- Gessmann, R., Kyvelidou, C., Papadovasilaki, M. & Petratos, K. (2011). *Biopolymers*, **95**, 202–207.
- Gessmann, R., Papadovasilaki, M., Drougkas, E. & Petratos, K. (2015). *Acta Cryst.* **F71**, 19–23.
- Hira, D., Nojiri, M. & Suzuki, S. (2009a). *Acta Cryst.* **D65**, 85–92.
- Hira, D., Nojiri, M. & Suzuki, S. (2009b). *Acta Cryst.* **F65**, 116–119.
- Impagliazzo, A., Blok, A. J., Cliff, M. J., Ladbury, J. E. & Ubbink, M. (2007). *J. Am. Chem. Soc.* **129**, 226–233.
- Inoue, T., Kai, Y., Harada, S., Kasai, N., Ohshiro, Y., Suzuki, S., Kohzuma, T. & Tobari, J. (1994). *Acta Cryst.* **D50**, 317–328.
- Inoue, T., Nishio, N., Kai, Y., Harada, S., Ohshiro, Y., Suzuki, S., Kohzuma, T., Shidara, S. & Iwasaki, H. (1993). *J. Biochem.* **114**, 761–762.
- Kakutani, T., Watanabe, H., Arima, K. & Beppu, T. (1981). *J. Biochem.* **89**, 463–472.
- Koshiyama, T., Shirai, M., Hikage, T., Tabe, H., Tanaka, K., Kitagawa, S. & Ueno, T. (2011). *Angew. Chem. Int. Ed.* **50**, 4849–4852.
- Laming, E. M., McGrath, A. P., Guss, J. M., Kappler, U. & Maher, M. J. (2012). *J. Inorg. Biochem.* **115**, 148–154.
- Laskowski, R. A., MacArthur, M. W., Moss, D. S. & Thornton, J. M. (1993). *J. Appl. Cryst.* **26**, 283–291.
- Lee, Y. J., Yi, H., Kim, W.-J., Kang, K., Yun, D. S., Strano, M. S., Ceder, G. & Belcher, A. M. (2009). *Science*, **324**, 1051–1055.
- Libeu, C. A. P., Kukimoto, M., Nishiyama, M., Horinouchi, S. & Adman, E. T. (1997). *Biochemistry*, **36**, 13160–13179.
- MacPherson, I. S., Rosell, F. I., Scofield, M., Mauk, A. G. & Murphy, M. E. P. (2010). *Protein Eng. Des. Sel.* **23**, 137–145.
- Margolin, A. L. & Navia, M. A. (2001). *Angew. Chem. Int. Ed.* **40**, 2204–2222.
- Maruccio, G., Biasco, A., Visconti, P., Bramanti, A., Pompa, P. P., Calabi, F., Cingolani, R., Rinaldi, R., Corni, S., Di Felice, R., Molinari, E., Verbeet, M. P. & Canters, G. W. (2005). *Adv. Mater.* **17**, 816–822.
- McGovern, R. E., Feifel, S. C., Lisdat, F. & Crowley, P. B. (2015). *Angew. Chem. Int. Ed.* **54**, 6356–6359.
- McPherson, A. & Gavira, J. A. (2014). *Acta Cryst.* **F70**, 2–20.
- Minton, A. P. (2006). *J. Cell Sci.* **119**, 2863–2869.
- Murphy, M. E. P., Turley, S. & Adman, E. T. (1997a). *J. Biol. Chem.* **272**, 28455–28460.
- Murphy, M. E. P., Turley, S. & Adman, E. T. (1997b). *Biological Electron Transfer Chains: Genetics, Composition and Mode of Operation*, edited by G. W. Canters & E. Vijgenboom, pp. 115–128. Dordrecht: Springer.
- Murshudov, G. N., Skubák, P., Lebedev, A. A., Pannu, N. S., Steiner, R. A., Nicholls, R. A., Winn, M. D., Long, F. & Vagin, A. A. (2011). *Acta Cryst.* **D67**, 355–367.
- Nojiri, M. (2016). *Metalloenzymes in Denitrification: Applications and Environmental Impacts*, edited by I. Moura, J. J. G. Moura, S. R. Pauleta & L. B. Maia, pp. 91–113. London: Royal Society of Chemistry. <https://doi.org/10.1039/9781782623762-00091>.
- Otwinowski, Z. & Minor, W. (1997). *Methods Enzymol.* **276**, 307–326.
- Petratos, K., Banner, D. W., Beppu, T., Wilson, K. S. & Tsernoglou, D. (1987). *FEBS Lett.* **218**, 209–214.
- Petratos, K., Dauter, Z. & Wilson, K. S. (1988). *Acta Cryst.* **B44**, 628–636.
- Petratos, K., Papadovasilaki, M. & Dauter, Z. (1995). *FEBS Lett.* **368**, 432–434.
- Prudêncio, M., Rohovec, J., Peters, J. A., Tocheva, E., Boulanger, M. J., Murphy, M. E. P., Hupkes, H. J., Kusters, W., Impagliazzo, A. & Ubbink, M. (2004). *Chem. Eur. J.* **10**, 3252–3260.
- Sato, K., Crowley, P. B. & Dennison, C. (2005). *J. Biol. Chem.* **280**, 19281–19288.
- Solomon, E. I., Szilagyi, R. K., DeBeer George, S. & Basumallick, L. (2004). *Chem. Rev.* **104**, 419–458.
- Tezcan, F. A., Crane, B. R., Winkler, J. R. & Gray, H. B. (2001). *Proc. Natl Acad. Sci. USA*, **98**, 5002–5006.
- Vagin, A. & Teplyakov, A. (2010). *Acta Cryst.* **D66**, 22–25.

- Vakoufari, E., Wilson, K. S. & Petratos, K. (1994). *FEBS Lett.* **347**, 203–206.
- Velarde, M., Huber, R., Yanagisawa, S., Dennison, C. & Messerschmidt, A. (2007). *Biochemistry*, **46**, 9981–9991.
- Vlasie, M. D., Fernández-Busnadiego, R., Prudêncio, M. & Ubbink, M. (2008). *J. Mol. Biol.* **375**, 1405–1415.
- Williams, P. A., Fülöp, V., Leung, Y.-C., Chan, C., Moir, J. W. B., Howlett, G., Ferguson, S. J., Radford, S. E. & Hajdu, J. (1995). *Nature Struct. Mol. Biol.* **2**, 975–982.
- Winn, M. D. *et al.* (2011). *Acta Cryst.* **D67**, 235–242.
- Yamaguchi, T., Akao, K., Takashina, A., Asamura, S. M., Unno, M., Szilagyi, R. K. & Kohzuma, T. (2016). *RSC Adv.* **6**, 88358–88365.