Biochemical, spectroscopic and X-ray structural analysis of deuterated multicopper oxidase CueO prepared from a new expression construct for neutron crystallography

Mahfuza Akter,^a Chika Inoue,^a Hirofumi Komori,^b Nana Matsuda,^c Takeshi Sakurai,^c Kunishige Kataoka,^c Yoshiki Higuchi^{a,d} and Naoki Shibata^{a,d*}

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^aDepartment of Life Science, Graduate School of Life Science, University of Hyogo, 3-2-1 Koto, Kamigori, Ako-gun, Hyogo 678-1297, Japan, ^bFaculty of Education, Kagawa University, 1-1 Saiwai-cho, Takamatsu, Kagawa 760-8522, Japan, ^cGraduate School of Natural Science and Technology, Kanazawa University, Kakuma, Kanazawa, Ishikawa 920-1192, Japan, and ^dRIKEN SPring-8 Center, 1-1-1 Koto, Sayo-cho, Hyogo 679-5148, Japan. *Correspondence e-mail: shibach@sci.u-hyogo.ac.jp

Multicopper oxidases oxidize various phenolic and nonphenolic compounds by using molecular oxygen as an electron acceptor to produce water. A multicopper oxidase protein, CueO, from *Escherichia coli* is involved in copper homeostasis in the bacterial cell. Although X-ray crystallographic studies have been conducted, the reduction mechanism of oxygen and the proton-transfer pathway remain unclear owing to the difficulty in identifying H atoms from X-ray diffraction data alone. To elucidate the reaction mechanism using neutron crystallography, a preparation system for obtaining large, high-quality single crystals of deuterated CueO was developed. Tiny crystals were obtained from the deuterated CueO initially prepared from the original construct. The X-ray crystal structure of the deuterated CueO showed that the protein contained an incompletely truncated signal sequence at the N-terminus, which resulted in the heterogeneity of the protein sample for crystallization. Here, a new CueO expression system that had an HRV3C cleavage site just after the signal sequence was constructed. Deuterated CueO from the new construct was expressed in cells cultured in deuterated algae-extract medium and the signal sequence was completely eliminated by HRV3C protease. The deuteration level of the purified protein was estimated by MALDI-TOF mass spectrometry to be at least 83.2% compared with nondeuterated protein. Nondeuterated CueO crystallized in space group $P2_1$, with unit-cell parameters $a = 49.51$, $b = 88.79$, $c = 53.95$ Å, $\beta = 94.24^\circ$, and deuterated CueO crystallized in space group $P2_12_12_1$, with unit-cell parameters $a = 49.91$, $b = 106.92$, $c = 262.89$ Å. The crystallographic parameters for the crystals of the new construct were different from those previously reported for nondeuterated crystals. The nondeuterated and deuterated CueO from the new construct had similar UV–Vis spectra, enzymatic activities and overall structure and geometry of the ligands of the Cu atoms in the active site to those of previously reported CueO structures. These results indicate that the CueO protein prepared using the new construct is suitable for further neutron diffraction studies.

1. Introduction

CueO is a protein in the multicopper oxidase family that is involved in copper homeostasis in *Escherichia coli* (Outten *et al.*, 2001; Rensing & Grass, 2003). It is used as the cathodic enzyme in biofuel cells in addition to in the copper-efflux system of *E. coli* (Komori *et al.*, 2012; Grass & Rensing, 2001). CueO contains four Cu atoms: a type I copper (T1 Cu) and a trinuclear copper centre (TNC) that consists of a type II copper (T2 Cu) and a pair of type III coppers (T3 Cu) (Komori *et al.*, 2012; Grass & Rensing, 2001; Solomon *et al.*,

1996). The T1 Cu site of CueO transfers electrons from the substrate to the TNC and reduces dioxygen to two water molecules using a four-electron reduction system (Sakurai & Kataoka, 2007; Solomon *et al.*, 2008). This four-electron reduction of dioxygen by multicopper oxidase is a crucial step, because no reactive oxygen species such as hydroxyl radicals or superoxide are generated by this reduction system (Kataoka *et al.*, 2009). Therefore, multicopper oxidase is promising for use as the cathodic enzyme in biofuel cells (Miura *et al.*, 2007; Tsujimura *et al.*, 2008; Miura *et al.*, 2009). Two reaction intermediates, known as intermediate I (peroxide intermediate) and intermediate II (native intermediate), have been found in this reduction system (Komori *et al.*, 2012; Sakurai & Kataoka, 2007; Bento *et al.*, 2005). However, the reaction mechanism of this reduction system is still not fully understood because there is no clear evidence to support the presence of peroxide at the TNC. Although X-ray crystal structures of CueO in various redox states have been determined, the reduction mechanism of the oxygen-transfer and proton-transfer pathway has not been clarified.

To understand the reaction mechanism of CueO, it is necessary to identify the protonation states of the active site and the hydrogen-bonding network surrounding the catalytic residues, which is possible using neutron crystallography (Kossiakov & Spencer, 1981). A completely deuterated protein crystal is preferred for neutron diffraction studies in order to avoid incoherent signals from the protein H atoms, which reduce the signal-to-noise ratio.

During the preparation of the deuterated CueO obtained from the original expression system, we found an incompletely truncated signal sequence at the N-terminus. This additional heterogeneous sequence may hamper the well ordered crystal packing of the molecules that is necessary for obtaining large single crystals suitable for neutron diffraction. In this work, we designed a new construct by adding a human rhinovirus 3C (HRV3C) protease cleavage site after the signal sequence, and we prepared deuterated CueO (CueO-d) without the signal sequence and its crystals in D₂O. We also obtained the enzymatic activity, UV-Vis spectra and X-ray crystal structures of nondeuterated CueO (CueO-h) and CueO-d from our construct and of deuterated CueO from the original construct (oCueO-d) to confirm that there were no major differences in the active site and overall structures caused by deuteration of the protein.

2. Materials and methods

2.1. Construction of the expression plasmid

The expression plasmid for C-terminally *Strep*-tagged wild-type CueO (Kataoka *et al.*, 2007; pASK-IBA3plus-wtCueO) was constructed using pASK-IBA3plus (IBA GmbH) linearized with BsaI restriction endonuclease. The expression plasmid for HRV3C-CueO carrying a modified Twin-Strep-tag followed by an HRV3C protease recognition site, inserted between residues Ala28 and Ala29, was constructed from the expression plasmid for wild-type CueO from *E. coli*. A

nucleotide fragment coding for the mature region of CueO was amplified by PCR using the primers HRV3C-GG-CueO_A29-f (5'-CTGTTCCAGGGGCCCCGGCGGTGCAGAACGCCCAACGTTACC-3') and CueO-stopstop_pASKIBA3-r (5'-CACAGGTCAAGCTTATTATACCGTAAACCCTAACATCATCC-3'), where underlined characters indicate overlap sequences assembled by In-Fusion (Clontech Laboratories) and Q5 Hot Start DNA polymerase (New England Biolabs). A fragment encoding the modified Twin-Strep-tag and HRV3C recognition sequences was obtained by PCR using the primers STreP-f (5'-TGGAGCCACCCGCAGTTC-3') and HRV3C-r (5'-GGGCCCCCTGGAACAGAACTTC-3') from a modified pCold vector (Takara Bio) containing a 6×His tag, a Twin-Strep-tag and the HRV3C recognition site (data not shown; Supplementary Table S1). The signal peptide region (Met1–Ala28) of CueO and the vector parts were amplified by inverse PCR using the primers stopstop_pASKIBA3-f (5'-TAATAAGCTTGACCTGTGAAGTG-3') and CueO_E30_STreP-r (5'-CTGCGGGTGGCTCCAACTACCGCCACTTGCCGCAATACTGCGCG-3') from pASK-IBA3plus-CueO. The resulting PCR products were assembled by using In-Fusion to generate a circular plasmid. Each plasmid was transformed into *E. coli* BL21 (DE3) cells.

2.2. Expression and purification of CueO

The *E. coli* BL21 (DE3) strain was used to express the CueO protein. Nondeuterated and deuterated CueO containing the inserted HRV3C sequence (HRV3C-CueO-h and HRV3C-CueO-d) were obtained from cells grown at 37°C in Luria–Bertani (LB) medium and deuterated algae extract (CELSTONE-d, Spectra Stable Isotopes) medium, respectively, containing 0.1 mg ml⁻¹ sodium ampicillin. Protein expression was induced by the addition of 0.05 µg ml⁻¹ anhydrotetracycline and 1 mM CuSO₄ at an OD₆₀₀ value of 0.5. After induction, the culture was incubated for 18 h at 25°C in the LB medium and at 37°C in the deuterated medium with shaking at 180 rev min⁻¹. The periplasmic fractions of the cells were prepared by cold osmotic shock using 20% (w/v) sucrose and 10 mM Tris–HCl pH 8.0. After centrifugation, the collected cells were suspended in 10 mM Tris–HCl pH 8.0 buffer containing protease-inhibitor cocktail (Roche) and sonicated to increase the yield of deuterated soluble protein. However, a sufficient yield of nondeuterated soluble protein was obtained by osmotic shock alone. The supernatant fractions containing the soluble proteins were applied onto a *Strep*-Tactin Sepharose affinity resin (IBA GmbH) column equilibrated with 50 mM Tris–HCl buffer pH 8.0. The same buffer was used to wash the column and HRV3C-CueO-d was eluted with the same buffer supplemented with 2.5 mM desthiobiotin. HRV3C-CueO-d was cleaved by an overnight reaction with the HRV3C enzyme at an enzyme:substrate ratio of 1:20, and the truncated signal sequence in the N-terminal part was removed by *Strep*-Tactin Sepharose affinity resin. Finally, CueO-d was purified by gel-filtration chromatography on a HiLoad 16/60 Superdex 200 column (GE Healthcare) pre-equilibrated with 20 mM sodium phosphate pH 6.0 buffer. In

contrast, after treatment with HRV3C, CueO-h was applied onto a 5 ml HiTrap Q HP column (GE Healthcare) equilibrated with 10 mM Tris-HCl pH 8.5 buffer and was eluted from the column with a linear gradient of NaCl (0–1.0 M) in the same buffer. The protein was further purified by gel-filtration chromatography using the same method as for CueO-d. oCueO-d was obtained from the deuterated medium without inserting the HRV3C sequence and was purified directly by affinity and gel-filtration chromatography with no enzymatic cleavage reaction. After the final purification step, the protein purity was determined by SDS-PAGE.

The final protein samples of CueO-d and oCueO-d were prepared by buffer exchange from nondeuterated (20 mM sodium phosphate pH 6.0 to deuterated (20 mM sodium phosphate pD 6.0) buffer solutions *via* repetitive concentration and dilution of the protein by centrifugation with a protein concentrator (30 kDa MWCO, Vivaspin 6, GE Healthcare). For CueO-h, the sample in 20 mM sodium phosphate pH 6.0 buffer was directly concentrated using the protein concentrator.

2.3. Absorption spectra

UV-Vis absorbance spectra were recorded at room temperature at 250–800 nm using a spectrophotometer (V-660, Jasco). The measurements were performed using a cell with a 1 cm path length and a protein concentration of 1 mg ml⁻¹.

2.4. Biochemical assay

The enzymatic activities of CueO-d, CueO-h and oCueO-d were calculated from the absorption increases at 420 nm ($\epsilon = 36\,000\text{ M}^{-1}\text{ cm}^{-1}$) at 25°C corresponding to the oxidized product of 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) catalyzed by CueO. Each reaction solution (1 ml) contained 6 mM ABTS, 1 mM CuSO₄ and 50 mM sodium acetate pH/pD 5.5 (or 5.1) buffer.

2.5. Mass spectrometry

MALDI-TOF mass analysis was performed using an ultrafleXtreme mass spectrometer (Bruker Daltonics). Purified samples (0.5 mg ml⁻¹) were mixed with Super-DHB (Sigma-Aldrich; 90:10 2,5-dihydroxybenzoic acid:2-hydroxy-5-methoxybenzoic acid), deposited on a ground steel plate and allowed to dry at room temperature. Mass calibration was performed using bovine serum albumin (66.4 kDa) and protein A (44.6 kDa). Mass spectra were obtained by the accumulation of 10 000 shots for each analysis and were processed using *FlexAnalysis* software (Bruker Daltonics).

2.6. Crystallization

Crystallization was performed by the sitting-drop vapour-diffusion method at 20°C. For CueO-d and oCueO-d (~6 mg ml⁻¹ protein in 20 mM sodium phosphate pD 6.0), 24-well sitting-drop Cryschem plates (Hampton Research) were used (protein:precipitant ratio of 1:1, 5 µl each). For CueO-h (~6 mg ml⁻¹ protein in 20 mM sodium phosphate pH

6.0), 96-well sitting-drop plates (Greiner Bio-One) were used (protein:precipitant ratio of 1:1, 1 µl each). PGA, PACT premier, ProPlex and Stura FootPrint Combination reagents (Molecular Dimensions) and the Wizard Precipitant Synergy and Wizard Classic reagents (Rigaku Reagents) were used for crystallization screening.

2.7. X-ray diffraction experiments and structure refinement

The X-ray diffraction data sets for oCueO-d and CueO-d were collected on the BL38B1 beamline, SPring-8, Japan using a Quantum 315 CCD detector (ADSC) and on the NE3A beamline, Photon Factory, Japan using a PILATUS 2M-F CCD detector (Dectris). The crystals were cryoprotected by flash-soaking in mother liquor supplemented with 15% CryoProtX solution No. 3 (Molecular Dimensions) for oCueO-d and 15% glycerol for CueO-d immediately before cooling to 100 K in a nitrogen-gas stream on the beamline. Diffraction data sets were indexed, integrated and scaled with *HKL-2000* (Otwinowski & Minor, 1997).

The structures were solved by the molecular-replacement method using the reported structure of CueO (PDB entry 1kv7; Roberts *et al.*, 2002) as a template. The individual atomic coordinates were refined by *PHENIX* (Adams *et al.*, 2010) and model building was performed with *Coot* (Emsley *et al.*, 2010). In the structures, water molecules were added automatically in *Coot* before being checked manually and refined. The atomic coordinates and structure factors were deposited in the Protein Data Bank with accession codes 5b7e for oCueO-d, 5b7m for CueO-d and 5b7f for CueO-h. The figures were prepared with *PyMOL* (v.1.3r1; Schrödinger).

Figure 1
SDS-PAGE analysis of wild-type oCueO-d (original construct) and HRV3C-CueO (new construct). (a) 10% SDS-PAGE for wild-type oCueO-d (original construct). Lane M, protein markers (labelled in kDa). Lane 1, purified oCueO-d protein with a partially truncated signal sequence at the N-terminus. (b) 10% SDS-PAGE for HRV3C-CueO (new construct). Lane M, protein markers (labelled in kDa). Lane 1, HRV3C-CueO protein. Lane 2, final purified CueO-d protein.

3. Results and discussion

3.1. Expression and purification

The expression temperature for HRV3C-CueO-h was 25°C; however, HRV3C-CueO-d did not express well at this temperature. Good expression was observed when the cells were grown in CELTONE-d medium at 37°C. Although the expression temperature was different, the total yields per litre from the two types of medium were similar ($\sim 1.5 \text{ mg l}^{-1}$ for CueO-h and 1.3 mg l^{-1} for CueO-d).

To maximize the recovery of the protein from the cells, osmotic shock and sonication were used for CueO-d, whereas only osmotic shock was sufficient for CueO-h. Affinity, ion-exchange and gel-filtration chromatography were required in order to obtain sufficiently purified CueO-h for crystallization. However, for CueO-d two chromatography steps were sufficient to obtain a crystallization-grade protein sample (Figs. 1*a* and 1*b*).

3.2. Absorption spectra

The UV-Vis spectra of oCueO-h, oCueO-d, CueO-h and CueO-d were similar (Supplementary Fig. S1), indicating that introduction of the HRV3C cleavage site did not alter the structure of the T1 Cu site of the enzyme.

3.3. Enzymatic activity

On comparing the enzymatic activities of oCueO-h, CueO-h and CueO-d, CueO-d showed a value that was lower than the others by less than 10% (Fig. 2). This is probably owing to the deuteration of the protein, but the negative influence of deuteration on the activity is small, suggesting that CueO-d prepared using the present method is as intact as oCueO-h and CueO-h.

3.4. Deuteration level determined by mass spectrometry

The mass spectra for CueO-d and CueO-h are shown in Supplementary Figs. S2(*a*) and S2(*b*), respectively. The molecular mass and the total number of H atoms in HRV3C-cleaved CueO-h were predicted by *Protein Calculator* v.3.4 (<http://protcalc.sourceforge.net/>; Table 1) to be 53 654 and 3729, respectively. The average mass value (53 752.7 Da) for three separate measurements for CueO-h was in good agreement with the predicted mass. The deuteration ratio of CueO-d can be estimated from the difference in mass between CueO-d and CueO-h. However, it is difficult to estimate the accurate ratio since CueO-d was purified in an H₂O-based buffer, in which the exchangeable D atoms are replaced by H atoms with a certain probability during purification. Of 3729 H atoms, 2932 are nonexchangeable C—H atoms, 337 are N—H or O—H atoms of side chains, which are likely to be exchangeable, and 460 are N—H atoms on the main chain, which would be partly exchangeable. Supposing that 30% of the main-chain H atoms are nonexchangeable, 138 of 460 main-chain D atoms remain in the protein. Thus, 3070 (2932 + 138) H atoms would be nonexchangeable. The difference in mass between CueO-d and CueO-h is 3104, indicating that the

Table 1

Summary of MALDI-TOF mass-spectrometry results.

Measurement	Peak (<i>m/z</i>)			
	CueO-d		CueO-h	
	[<i>M</i> + H] ⁺	[<i>M</i> + 2H] ²⁺	[<i>M</i> + H] ⁺	[<i>M</i> + 2H] ²⁺
1	56811.0	28415.8	53690.7	26834.4
2	56880.8	28445.0	53821.8	26914.6
3	56878.3	28438.4	53745.7	26874.7
Average	56856.7	28433.1	53752.7	26874.6
Difference between CueO-d and CueO-h	3104.0	1558.5	—	—

deuteration ratio is almost 100%, if the effect of the back-replacement of deuterium after purification is ignored. Supposing that all the exchangeable H atoms were back-replaced by deuterium in the deuterated buffer solution, the deuteration ratio is calculated to be 83.2%.

3.5. Crystallization

The original crystallization condition for oCueO-h was 20% (w/v) PEG 6000, 0.2 M lithium chloride, 0.1 M Na HEPES pH 7.0. For CueO-d, we obtained crystals (Supplementary Fig. S3) in a similar condition [20% (w/v) PEG 6000, 0.2 M lithium chloride, 0.1 M HEPES pH 7.0]. However, in the case of oCueO-d and CueO-h we could not obtain good-quality crystals under this condition. We then screened other crystallization conditions and obtained good crystals of oCueO-d in a solution consisting of 14% (w/v) PEG 8000, 0.1 M Na HEPES pH 7.0 and CueO-h crystals in 20% (w/v) PEG 6000, 0.2 M calcium chloride, 0.1 M MES pH 6.0. By introducing an HRV3C cleavage site just after the signal sequence, we obtained crystals of the nondeuterated and deuterated enzymes that diffracted to high resolution. For CueO-d, we obtained crystals that were thicker than those of oCueO-d.

Figure 2

Enzymatic activity of oCueO-h, CueO-h and CueO-d. The activities are shown as mean values with standard deviations calculated from triplicate experiments. Specific activities are expressed as units of activity per milligram of protein, where one activity unit represents the oxidation of 1 μmol of ABTS per minute. Error bars indicate the mean standard deviation of three independent measurements.

Table 2
Data-collection and refinement statistics.

Values in parentheses are for the highest resolution shell.

	oCueO-d	CueO-h	CueO-d
Data collection			
Diffraction source	BL38B1, SPring-8	NE3A, Photon Factory	NE3A, Photon Factory
Wavelength (Å)	1.0000	1.0000	1.0000
Space group	$P2_12_12_1$	$P2_1$	$P2_12_12_1$
a, b, c (Å)	52.32, 70.42, 134.03	49.51, 88.79, 53.95	49.91, 106.92, 262.89
α, β, γ (°)	90, 90, 90	90, 94.24, 90	90, 90, 90
Resolution range (Å)	50.0–1.42 (1.44–1.42)	50.0–1.45 (1.48–1.45)	50.0–1.80 (1.83–1.80)
$R_{\text{merge}}^{\dagger}$ (%)	5.2 (98.6)	10.8 (60.1)	12.1 (88.4)
$R_{\text{p.i.m.}}$ (%)	2.2 (42.7)	4.6 (28.3)	5.2 (40.7)
$CC_{1/2}$	0.992 (0.709)	0.985 (0.788)	0.982 (0.732)
$\langle I/\sigma(I) \rangle^{\ddagger}$	31.4 (1.76)	15.9 (2.29)	15.1 (1.98)
Completeness (%)	100.0 (100.0)	99.8 (96.8)	99.7 (98.1)
Multiplicity	6.3 (6.2)	6.3 (5.0)	6.4 (5.4)
Total No. of reflections	593273	522228	834703
No. of unique reflections	94351 (4666)	82355 (3974)	131300 (6359)
Overall B factor from Wilson plot (Å ²)	15.5	12.7	21.5
Refinement			
Resolution (Å)	31.2–1.42 (1.45–1.42)	35.1–1.45 (1.48–1.45)	37.5–1.80 (1.85–1.80)
R_{work}	0.156 (0.242)	0.153 (0.227)	0.166 (0.250)
R_{free}	0.183 (0.265)	0.181 (0.285)	0.204 (0.283)
No. of non-H atoms (protein)	4282	4154	11511
Mean B value (Å ²)	22.70	17.80	30.00
No. of waters	558	485	763
R.m.s.d., bond lengths (Å)	0.007	0.006	0.007
R.m.s.d., bond angles (°)	1.15	1.14	1.10
Ramachandran plot			
Residues in favoured regions (%)	97	97	97
Residues in allowed regions (%)	3	3	3
Residues in disallowed regions (%)	0	0	0

$\dagger R_{\text{merge}} = \sum_{hkl} \sum_i |I_i(hkl) - \langle I(hkl) \rangle| / \sum_{hkl} \sum_i I_i(hkl)$, where $I_i(hkl)$ is the intensity of the i th observation. $\ddagger$ Although the mean $\langle I/\sigma(I) \rangle$ values for the highest resolution shell of oCueO-d and CueO-d are less than 2.0, the highest resolution cutoff was determined based on the $CC_{1/2}$ criterion proposed by Karplus & Diederichs (2012) and the completeness. In the highest resolution shells, the $CC_{1/2}$ and completeness values are greater than 0.7 and 95% in all data sets.

CueO-h crystallized in space group $P2_1$, with unit-cell parameters $a = 49.51$, $b = 88.79$, $c = 53.95$ Å, $\beta = 94.24^\circ$, like other previously reported nondeuterated wild-type CueO crystals (Komori *et al.*, 2012, 2013, 2014; Sakurai & Kataoka, 2007; Roberts *et al.*, 2003; Singh *et al.*, 2011). However, CueO-d crystallized in space group $P2_12_12_1$, with unit-cell parameters $a = 49.91$, $b = 106.92$, $c = 262.89$ Å. oCueO-d crystallized in space group $P2_12_12_1$, with unit-cell parameters $a = 52.32$, $b = 70.42$, $c = 134.0$ Å. The data-collection and refinement statistics for these three crystals are summarized in Table 2.

3.6. X-ray structures of oCueO-d, CueO-d and CueO-h

The structures of oCueO-d, CueO-h and CueO-d were solved at 1.42, 1.40 and 1.80 Å resolution, respectively. The values of R_{work} , R_{free} and other geometrical statistics showed that the structures were well refined (Table 2). Structural comparison of the CueO structures (Fig. 3) showed that there was no significant difference in the overall structure except for the N-terminal region of oCueO-d, which was not observed in CueO (PDB entry 1kv7), CueO-d and CueO-h. The N-terminus of oCueO-d contained extra residues (RAVF-AAE–) derived from the signal sequence. These extra residues may have affected the crystal packing and prevented large single crystals suitable for neutron diffraction from forming. Indeed, in the oCueO-d crystal the main-chain N atom of Arg24, which is the first residue of the extra residues, forms a

Figure 3
Overall structure of CueO. T1 copper ions are shown as dark blue spheres, T2/T3 copper ions are shown as gold spheres and water molecules at the TNC are shown as red spheres. The previously published nondeuterated CueO structure (PDB entry 1kv7, grey), oCueO-d (orange), CueO-h (magenta) and CueO-d (green) are superimposed. The overall structures of CueO are almost identical, but the deuterated oCueO-d contains an extra seven residues at the N-terminus (shown in stick representation).

Figure 4

The copper centres of CueO: T1 copper site (a) and T2/T3 copper site (b). CueO (PDB entry 1kv7, grey), oCueO-d (orange), CueO-h (magenta) and CueO-d (green) are superimposed.

hydrogen bond to the side chain of Asp332 of the adjacent molecule. In contrast, there is no significant interaction between protein molecules around the N-terminus in the CueO-d crystal. The extra residues are likely to be attributable to inhibition of the cleavage of the signal sequence by the deuteration of oCueO. The mature CueO-h was easily extracted from the cells by osmotic shock because it was mainly located in the periplasmic space. The yield of oCueO-d obtained by osmotic shock was low; thus, it was extracted by sonication, suggesting that the bacterial cells did not contain large amounts of mature oCueO-d in the periplasmic space. As a result, we obtained a small amount of mature (signal-truncated), partially truncated and untruncated premature oCueO-d by sonication from the periplasmic and cytoplasmic spaces during enzyme preparation (Supplementary Fig. S4). The mechanism of inhibition of the cleavage of the enzymatic signal sequence by deuteration was unclear.

3.7. Structure of the copper centres

CueO contains four copper ions, arranged as a T1 site (one ion) and a T2 (one ion)/T3 (two ions) cluster. The T1 Cu is coordinated by His443, His505, Cys500 and Met510. The T2 Cu is coordinated by His101 and His446, whereas the two T3 Cu ions are coordinated by His103, His141 and His501, by His143, His448 and His499, and by a water molecule.

Superimposing CueO (PDB entry 1kv7), oCueO-d, CueO-h and CueO-d demonstrates that despite the deuteration of the enzyme, the T1 (Fig. 4a) and T2/T3 Cu clusters (Fig. 4b) are identical, with average root-mean-square deviations of 0.08 and 0.12 Å, respectively. The distances between the copper ions and the ligand atoms (Supplementary Table 2) indicate that deuteration of CueO does not alter the coordination geometry of the active site of the protein.

4. Conclusions

We established an expression system for deuterated CueO with an HRV3C cleavage site just after the signal sequence;

the deuterated protein obtained from the original system had seven extra residues originating from the signal sequence. We developed an efficient purification procedure for CueO-d. Mass spectrometry showed that the CueO samples were highly purified and deuterated, indicating that this is a candidate protein for obtaining sufficiently large, high-quality crystals for neutron diffraction studies. CueO-h and CueO-d from the new construct and oCueO-d from the original construct were crystallized and their structures were solved by X-ray crystallography. The overall and active-site X-ray crystal structures and the spectroscopic and biochemical data showed that CueO-d and CueO-h had the same features as oCueO-h. Based on the biochemical and physiochemical data, the structure–function relationship of CueO is expected to be elucidated by neutron crystallography of the present CueO sample.

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References

- Adams, P. D. *et al.* (2010). *Acta Cryst.* **D66**, 213–221.
- Bento, I., Martins, L. O., Lopes, G. G., Carrondo, M. A. & Lindley, P. F. (2005). *Dalton Trans.*, pp. 3507–3513.

- Emsley, P., Lohkamp, B., Scott, W. G. & Cowtan, K. (2010). *Acta Cryst.* **D66**, 486–501.
- Grass, G. & Rensing, C. (2001). *Biochem. Biophys. Res. Commun.* **286**, 902–908.
- Karplus, P. A. & Diederichs, K. (2012). *Science*, **336**, 1030–1033.
- Kataoka, K., Komori, H., Ueki, Y., Konno, Y., Kamitaka, Y., Kurose, S., Tsujimura, S., Higuchi, Y., Kano, K., Seo, D. & Sakurai, T. (2007). *J. Mol. Biol.* **373**, 141–152.
- Kataoka, K., Sugiyama, R., Hirota, S., Inoue, M., Urata, K., Minagawa, Y., Seo, D. & Sakurai, T. (2009). *J. Biol. Chem.* **284**, 14405–14413.
- Komori, H., Kajikawa, T., Kataoka, K., Higuchi, Y. & Sakurai, T. (2013). *Biochem. Biophys. Res. Commun.* **438**, 686–690.
- Komori, H., Sugiyama, R., Kataoka, K., Higuchi, Y. & Sakurai, T. (2012). *Angew. Chem. Int. Ed.* **51**, 1861–1864.
- Komori, H., Sugiyama, R., Kataoka, K., Miyazaki, K., Higuchi, Y. & Sakurai, T. (2014). *Acta Cryst.* **D70**, 772–779.
- Kossiakoff, A. A. & Spencer, S. A. (1981). *Biochemistry*, **20**, 6462–6474.
- Miura, Y., Tsujimura, S., Kamitaka, Y., Kurose, S., Kataoka, K., Sakurai, T. & Kano, K. (2007). *Chem. Lett.* **36**, 132–133.
- Miura, Y., Tsujimura, S., Kurose, S., Kamitaka, Y., Kataoka, K., Sakurai, T. & Kano, K. (2009). *Fuel Cells*, **9**, 70–78.
- Otwinowski, Z. & Minor, W. (1997). *Methods Enzymol.* **276**, 307–326.
- Outten, F. W., Huffman, D. L., Hale, J. A. & O'Halloran, T. V. (2001). *J. Biol. Chem.* **276**, 30670–30677.
- Rensing, C. & Grass, G. (2003). *FEMS Microbiol. Rev.* **27**, 197–213.
- Roberts, S. A., Weichsel, A., Grass, G., Thakali, K., Hazzard, J. T., Tollin, G., Rensing, C. & Montfort, W. R. (2002). *Proc. Natl Acad. Sci. USA*, **99**, 2766–2771.
- Roberts, S. A., Wildner, G. F., Grass, G., Weichsel, A., Ambrus, A., Rensing, C. & Montfort, W. R. (2003). *J. Biol. Chem.* **278**, 31958–31963.
- Sakurai, T. & Kataoka, K. (2007). *Cell. Mol. Life Sci.* **64**, 2642–2656.
- Singh, S. K., Roberts, S. A., McDevitt, S. F., Weichsel, A., Wildner, G. F., Grass, G. B., Rensing, C. & Montfort, W. R. (2011). *J. Biol. Chem.* **286**, 37849–37857.
- Solomon, E. I., Augustine, A. J. & Yoon, J. (2008). *Dalton Trans.*, pp. 3921–3932.
- Solomon, E. I., Sundaram, U. M. & Machonkin, T. E. (1996). *Chem. Rev.* **96**, 2563–2606.
- Tsujimura, S., Miura, Y. & Kano, K. (2008). *Electrochim. Acta*, **53**, 5716–5720.