STRUCTURAL BIOLOGY
COMMUNICATIONS

ISSN 2053-230X

Exogenous acetate ion reaches the type II copper centre in CueO through the water-excretion channel and potentially affects the enzymatic activity

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Received 12 April 2016

Accepted 7 June 2016

Edited by I. Tanaka, Hokkaido University, Japan

Keywords: bioinorganic chemistry; enzyme catalysis; metalloproteins; CueO; multicopper oxidase.

PDB reference: CueO (citrate buffer), 4ef3

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

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The acetate-bound form of the type II copper was found in the X-ray structure of the multicopper oxidase CueO crystallized in acetate buffer in addition to the conventional OH[−]-bound form as the major resting form. The acetate ion was retained bound to the type II copper even after prolonged exposure of a CueO crystal to X-ray radiation, which led to the stepwise reduction of the Cu centres. However, in this study, when CueO was crystallized in citrate buffer the OH[−]-bound form was present exclusively. This fact shows that an exogenous acetate ion reaches the type II Cu centre through the water channel constructed between domains 1 and 3 in the CueO molecule. It was also found that the enzymatic activity of CueO is enhanced in the presence of acetate ions in the solvent water.

1. Introduction

Multicopper oxidase (MCO) belongs to a class of copper enzymes that harbour four Cu atoms classified as type I copper (T1Cu), type II copper (T2Cu) and type III copper (T3Cu) (Komori & Higuchi, 2010, 2015; Sakurai & Kataoka, 2011). MCOs oxidize a variety of substrates such as phenols, aromatic amines and transition-metal ions at T1Cu, and reduce O₂ at the trinuclear copper centre (TNC), which is comprised of one T2Cu atom and a pair of T3Cu atoms. During the four-electron reduction of O₂ to H₂O by MCOs, activated oxygen species are not formed or are not released from the TNC if they are formed. Therefore, MCOs have been used in biofuel cells as a cathodic enzyme in addition to uses in clinical tests and for pigment formation (Miura *et al.*, 2009).

In the resting form of MCOs, T1Cu and T2Cu can be detected by electron paramagnetic resonance (EPR) spectroscopy, but T3Cu atoms are not detected owing to anti-ferromagnetic interaction between them. This classical resting form of the TNC, in which the T3Cu atoms are bridged by only an OH[−] ion, was first observed in the crystal structure of ascorbate oxidase and has subsequently been found in other MCOs (Messerschmidt *et al.*, 1989). However, variants with an O-centred TNC structure as an intermediate in the O₂-reduction process (Huang *et al.*, 1999; Komori *et al.*, 2012; Morishita *et al.*, 2014) and partly reduced TNC structures have recently been discovered in resting MCOs and mutants (Polyakov *et al.*, 2009). Further, an exogenous Cl[−]-binding TNC has also been reported, presumably because crystallization had been performed in the presence of this small

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anion (Hakulinen *et al.*, 2002). The binding of exogenously soaked azide and peroxide to the TNC have also been reported for ascorbate oxidase, although discussion of the O_2 -reduction mechanism has become more complicated owing to their diverse binding modes (Messerschmidt *et al.*, 1993).

As a result of studies on plant laccase (Huang *et al.*, 1999; Zoppellaro *et al.*, 2001), CueO (Kataoka *et al.*, 2009), bilirubin oxidase (Kataoka *et al.*, 2005), Fet3p (Augustine *et al.*, 2007) and CotA (Roberts *et al.*, 2002; Mizutani *et al.*, 2010; Taylor *et al.*, 2005; Enguita *et al.*, 2003), it has been revealed that acidic amino acids play a vital role in the transport of H^+ and the tuning of the redox potential of copper centres by forming a hydrogen bond to the ligand groups and/or specific water molecules. Glu506 involved in the hydrogen-bond network leading from solvent water to the OH^- ion bridged between the T3Cu atoms in CueO (greenish-blue C^α atom and side chain shown as a ball-and-stick model and the greenish-blue

Figure 1

Tunnels leading from the protein surface to the TNC in CueO. The greenish-blue tunnel is the H^+ -transport channel, in which specific water molecules are present. At the end of the hydrogen-bond network the side chain and C^α of Glu506 (greenish-blue ball-and-stick representation) are located to form a hydrogen bond to the OH^- bridged between the T3Cu atoms *via* a water molecule. The purple tunnel constructed between domain 1 and domain 3 is for the excretion of water. Near its end, the side chain and C^α of Asp112 (purple ball-and-stick representation) are located. The role of the grey tunnel is not known, but it might be involved in the introduction of O_2 . The red arrows indicate the directions of flow of H^+ , O_2 and H_2O *via* the greenish-blue, grey and purple tunnels, respectively, and of e^- from T1Cu to the TNC *via* the His-Cys-His triad that connects them. Blue, green and red spheres are the T1Cu, T2Cu and T3Cu atoms, respectively. Tunnels are depicted using *Caver Analyst* (Kozlikova *et al.*, 2014) with the previously published CueO coordinates (PDB entry 4e9s).

Table 1

Crystallization conditions for the acetate-free CueO.

Method	Sitting-drop vapour diffusion
Plate type	24-well sitting-drop plate (Cryschem plate, Hampton Research)
Temperature (K)	293
Protein concentration (mg ml ⁻¹)	12
Buffer composition of protein solution	100 mM phosphate buffer pH 6.0
Composition of reservoir solution	100 mM sodium citrate buffer pH 5.6, 22% PEG 4000, 5% 2-propanol
Volume and ratio of drop (μl)	2:2
Volume of reservoir (μl)	500

tunnel in Fig. 1) is involved in the transport of H^+ to O_2 (Iwaki *et al.*, 2010; Komori *et al.*, 2013). On the other hand, Asp112 located in the channel constructed between domain 1 and domain 3 in the CueO molecule (purple C^α atom and side chain shown as a ball-and-stick model and the purple tunnel in Fig. 1) has been considered to be involved in the activation of O_2 and the excretion of H_2O (Ueki *et al.*, 2006; Kataoka *et al.*, 2009).

In a series of crystal structure analyses of CueO under low to high X-ray dose conditions (Komori *et al.*, 2014), we showed changes in the structure of the TNC from data set 1 to data set 6. It was apparent that the acetate-bound form was also present throughout data set 1 to data set 6 in addition to the conventional OH^- -bound resting form of T2Cu. In this communication, we determined the acetate-free CueO structure (PDB entry 4ef3) and compared it with the previously published coordinates (PDB entry 4e9s; Kataoka *et al.*, 2007), for which the presence of the acetate ion had not been discussed. Acetate ions easily reach T2Cu through the water channel, as demonstrated by the CueO crystals formed in the presence and absence of acetate ions.

2. Materials and methods

The purification and crystallization of CueO were performed according to previously reported methods (Kataoka *et al.*, 2007). The oxidizing activity of 2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) was measured as reported in Kataoka *et al.* (2007) except for increasing additions of ammonium acetate. Citrate buffer was used for crystallization instead of acetate buffer (Table 1).

For data collection, CueO crystals were soaked in a cryo-protectant solution (10% glycerol) for a few minutes prior to cooling under a cold stream of nitrogen. X-ray diffraction data sets were collected at 100 K on beamline BL26B2 ($\lambda = 0.8000 \text{ \AA}$ at SPring-8 using a MAR Research MX225 CCD detector (Ueno *et al.*, 2006).

The unit-cell parameters were determined and reflections were integrated using *HKL-2000* (Otwinowski & Minor, 1997) and the *CCP4* program package (Winn *et al.*, 2011). Using the reported structure of CueO (PDB entry 4ner; Komori *et al.*, 2014), the molecular-replacement method was carried out. Further model building and structure refinement were performed using *Coot* (Emsley *et al.*, 2010), *REFMAC* (Murshudov *et al.*, 2011) and *SHELX* (Sheldrick, 2008). The

progress and validity of the refinement process were checked by monitoring the R_{free} value for 5% of the total reflections (Brünger, 1992). The data-collection and refinement statistics are summarized in Table 2. Model geometry was analysed using *MolProbity* (Chen *et al.*, 2010) and no residues were found in the disallowed region of the Ramachandran plot. The figures were prepared using *PyMOL* (<http://www.pymol.org>). The coordinates have been deposited in the PDB with accession code 4ef3.

Tunnels leading from the protein surface to the TNC were visualized using *Caver Analyst* (Kozlikova *et al.*, 2014).

3. Results and discussion

In the previous structural analyses of CueO crystallized in acetate buffer we have shown changes in the structure around the TNC using six diffraction data sets collected under increasing X-ray dose conditions (PDB entries 4ner, 4e9q, 4e9r, 4e9s and 4e9t for data sets 1, 2, 4, 5 and 6, respectively; data set 3 was not deposited; Komori *et al.*, 2014). The TNC for data set 1 obtained under low X-ray dose conditions had the O-centred structure with OH^- -bridged T3Cu atoms and an OH^- -bound T2Cu ($\sim 50\%$). However, it was apparent that the acetate-bound T2Cu form was also present in place of OH^- ($\sim 50\%$; the presence of acetate ion was not mentioned in the previous crystal structure analyses). Fig. 2(a) shows the $2F_o - F_c$ map around T2Cu contoured at 1.0σ for acetate data set 1 (PDB entry 4ner) and Fig. 2(b) shows the $F_o - F_c$ map around T2Cu contoured at 4σ for the same data set. Residual

Table 2

Data collection and processing for the acetate-free CueO crystal.

Values in parentheses are for the outer shell.

Diffraction source	BL26B2, SPring-8
Wavelength (Å)	0.8000
Temperature (K)	100
Detector	MAR Research MX225
Crystal-to-detector distance (mm)	200.0
Rotation range per image (°)	1
Total rotation range (°)	180
Exposure time per image (s)	1
Attenuator	Al, 1000 μm
Space group	$P2_1$
a, b, c (Å)	50.28, 93.24, 59.59
α, β, γ (°)	90.00, 112.87, 90.00
Resolution range (Å)	17.60–1.90 (1.97–19.0)
No. of unique reflections	38421 (3579)
Completeness (%)	98.7 (92.1)
Multiplicity	3.7 (2.9)
$\langle I/\sigma(I) \rangle$	10.4 (2.0)
$R_{\text{r.i.m.}}^\dagger$	0.094 (0.343)
R	0.154 (0.213)
R_{free}	0.184 (0.254)

$^\dagger R_{\text{r.i.m.}}$ was estimated by multiplying the conventional R_{merge} value by the factor $[N/(N-1)]^{1/2}$, where N is the data multiplicity.

density is observable around the OH^- -bound T2Cu, indicating the coordination of an acetate ion in the place of OH^- . Fig. 3(a) shows the whole active site with the superimposition of forms representing both the binding of OH^- and acetate ion. The distances between the cupric T2Cu and the two O atoms derived from the acetate ion are 2.2 and 3.1 Å, indicating monodentate coordination by the deprotonated

Figure 2

(a) $2F_o - F_c$ map contoured at 1.0σ for acetate data set 1 (PDB entry 4ner). (b) $F_o - F_c$ map contoured at 4.0σ for acetate data set 1 under low X-ray dose conditions (PDB entry 4ner). (c) $2F_o - F_c$ map contoured at 1.0σ for acetate data set 5 under high X-ray dose conditions (PDB entry 4e9s). (d) OMIT map of acetate contoured at 4.0σ for acetate data set 5 (PDB entry 4e9s). T2Cu and O atoms are shown as green and red spheres, respectively.

hydroxyl group. The former bond distance is practically the same as that for the inorganic OH^- -bound form, although the distances of the $\text{Cu}^{2+}-\text{O}^-$ bond (for OH^- and CH_3CO_2^-) were reported to be considerably shorter at $<2\text{ \AA}$ in small-molecule studies (Bazhanova *et al.*, 2008).

The two other O species bound to Cu^{2+} are the central O^{2-} inside the copper triangle and the OH^- ion bridged between the T3Cu atoms (Fig. 3*a*), both of which originate from the O_2 co-substrate. Therefore, data set 1 collected under low X-ray dose conditions mainly contains a fully oxidized form of copper with O^{2-} at the centre of the TNC: the intermediate II form or the native intermediate (the *in situ*-measured *K*-edge spectrum indicated that all Cu centres were cupric). With regard to the coordination geometry of T2Cu, the square-planar structure with the 2N2O ligand atom set (two N atoms from two His residues and two O atoms from the central O^{2-} and OH^- or acetate) is typical of cupric ion and differs from the T-shaped structure without the central O^{2-} found in the classical resting form of MCOs.

Stepwise reductions of T1Cu, T2Cu and one of the T3Cu atoms (T3Cu_a) took place during the transformation from

data set 1 to data set 6. Fig. 3(*b*) indicates the structural change induced at the TNC. The stepwise reduction of T2Cu and T3Cu_a were accompanied by protonation of the central O^{2-} and the OH^- bound to T2Cu and the subsequent elimination of an H_2O molecule from the TNC through the water channel involving Asp112. Concomitantly, the $d-\pi$ interaction between T3Cu_a and Trp139 became stronger owing to a decrease in the distance between T3Cu_a and the edge of the indole ring.

The acetate-bound form of T2Cu was present throughout all of the data sets. Figs. 2(*c*) and 2(*d*) show the $2F_o - F_c$ map contoured at 1.0σ for acetate in data set 5 (PDB entry 4e9s) and the OMIT map of acetate contoured at 4.0σ , respectively. The distances between the reduced T2Cu and the two O atoms from the acetate moiety were 2.5 and 3.6 $\text{\AA}$. According to a small-molecule study, the bond distance between Cu^+ ion and the O atom of acetate ion is $\sim 1.9\text{ \AA}$ regardless of whether the structure is dimeric or tetrameric (Bazhanova *et al.*, 2008). The binding of a protonated acetic acid molecule by Cu^+ or Cu^{2+} has not been reported in small-molecule studies. A cage effect provided by a protein molecule might allow the presence of a protonated acetic acid near a cuprous ion, for which the geometry is linear (2N coordination) or T-shaped (2N1O coordination).

We have always utilized 100 mM ammonium acetate pH 5.2 with 20% polyethylene glycol (PEG 4000) and 5% 2-propanol to crystallize CueO and its mutants, which led to the formation of the acetate-bound form. To obtain unequivocal evidence that the acetate ion was derived from the buffer solution used for crystallization, we utilized a novel crystallization condition using citrate buffer at pH 5.6. As expected, the acetate-bound form was not observed even as a minor content in the crystals formed in citrate buffer (Fig. 4; PDB entry 4ef3; crystallographic data are given in Tables 1 and 2). The coordinates of all atoms in Fig. 5 were practically the same as those in Fig. 3(*a*) except that acetate ion was never observed. The

Figure 3
The active site of CueO crystallized in acetate buffer with OH^- and acetate-bound T2Cu. (*a*) Acetate data set 1 under low X-ray dose conditions (PDB entry 4ner). (*b*) Acetate data set 5 under high X-ray dose conditions (PDB data 4e9t). Both the OH^- and the acetate ion are shown together in (*a*) and (*b*). The central O atom is present in the centre of the TNC in (*a*), but has disappeared in (*b*), in which T2Cu and T3Cu_a are reduced by hydrated electrons. The large blue, green and orange spheres are the T1Cu, T2Cu and T3Cu atoms, respectively. The small red spheres are O^{2-} , OH^- or H_2O .

Figure 4
 $2F_o - F_c$ map contoured at 1.0σ for the citrate data (PDB entry 4ef3). T2Cu and O atoms are shown as green and red spheres, respectively.

presence of acetate ion was not mentioned in the previous crystal structure analyses.

In the early studies on MCOs such as plant laccase, ascorbate oxidase and ceruloplasmin, exogenous small anions such as azide and fluoride were utilized to study the properties of the TNC (Koudelka & Ettinger, 1988). There is no doubt that these anions reach the TNC, in particular T2Cu, easily. It has also been supposed that the H₂O molecule bound to T2Cu is deprotonated depending on the pH. T2Cu has frequently been observed to be depleted in many MCOs, suggesting that this copper centre is most easily accessible to solvent water among the four copper centres. Fig. 1 shows the presence of three channels leading from the protein surface to the TNC. The purple tunnel starting at the OH[−] coordinated to T2Cu is located between domain 1 and domain 3. Asp112 positioned

Figure 5

The structure of the copper centre in CueO crystallized in citrate buffer without an acetate ion bound to T2Cu (PDB entry 4ef3). The crystals for X-ray diffraction were obtained in citrate buffer pH 5.6, in contrast to those used for Fig. 1, which were obtained in acetate buffer pH 5.2. The central O atom is present in the centre of the TNC. The large blue, green and orange spheres are the T1Cu, T2Cu and T3Cu atoms, respectively. The small red spheres are O^{2−}, OH[−] or H₂O.

Figure 6

Effect of the addition of ammonium acetate on the oxidizing activity for 2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) of CueO in 0.1 M phosphate buffer pH 6 at 25°C as determined from absorption changes at 420 nm. One unit of activity is the amount of enzyme required to oxidize 1 μmol substrate per minute. The pH values of the reaction mixtures were not changed on the addition of ammonium acetate. Three data points were averaged for each specific activity.

to the side of it is hydrogen-bonded to the OH[−] ion coordinated to T2Cu *via* a water molecule and is also hydrogen-bonded to His101 and His448 coordinated to T2Cu and T3Cu_b, respectively. Mutation of Asp112 led to a reduction in enzymatic activity, indicating that this amino acid is one of the key amino acids in controlling the processes of O₂ reduction and H₂O excretion (Ueki *et al.*, 2006). The purple tunnel has been considered to play a role in the excretion of H₂O formed by the reduction of O₂. Although we have not performed a soaking experiment with acetate, we conclude that the acetate ion in Fig. 2 has come from the buffer solution. The hydrogen-bond wire formed by water molecules and Asp112 is considerably dynamic and allows exogenous acetate ion, which is bulkier than linear anions such as N₃[−] and O₂^{2−}, to reach the TNC and replace OH[−] or H₂O coordinated to T2Cu (see above).

Exogenous acetate ions reach the TNC through the purple tunnel in Fig. 1 and presumably also approach the entrance of the tunnel to transport H⁺ (greenish-blue tunnel). Since Asp112 and Glu506 with a carboxy group in their side chains are involved in the former and latter tunnels, respectively, we determined the enzymatic activity of CueO in phosphate buffer in the presence of 0–500 mM ammonium acetate. The oxidizing activity of CueO for ABTS was enhanced by the addition of up to 300 mM acetate ion, although it decreased at higher concentrations of acetate ion, presumably owing to the solution becoming slightly turbid (Fig. 6). In contrast to azide and halide ions, acetate ion is labile to substitution and does not inhibit the binding of O₂ at the TNC. Furthermore, acetate ion might assist the transport of proton to TNC by existing in and near the channels leading from solvent to TNC. Further studies are required to ascertain whether this hypothetical supporting effect of proton transport by acetate ion prevails more widely over other MCOs.

4. Conclusions

The present results indicate that the channel formed between domains 1 and 3 in the CueO molecule is sufficiently wide and dynamic to allow exogenous acetate ions to approach T2Cu deeply buried inside the CueO molecule. An effect of addition of acetate ion in enhancing the enzymatic activity of CueO has also been observed.

Acknowledgements

The present study was supported by Grants-in-Aid from Gakushin, Japan to TS (Nos. 26288076 and 2665031), a grant from the Inamori Foundation to HK and the fund for Kagawa University Young Scientists 2013 and 2015 (HK). We also thank the beamline staff at BL26B2 for their kind help with X-ray data collection.

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